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Agonist pharmacology of the neuronal α7 nicotinic receptor expressed in *Xenopus* oocytes

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The potencies and efficacies of seven agonists at chick α7 nicotinic receptors expressed in *Xenopus* oocytes were determined by whole cell recording. (+)-Anatoxin-a was the most potent agonist (EC_{50} = 0.58 μM) and acetylcholine was the least potent (EC_{50} = 320 μM). The rank order of agonist potencies was: (+)-anatoxin-a >> cytisine > (−)-nicotine > (+)-nicotine > DMPP > 1-acetyl-4-methylpiperazine methiodide > acetylcholine. DMPP evoked only very small currents: comparison of maximally effective agonist concentrations showed that DMPP was only one-fifth as efficacious as other agonists. Previously published IC_{50} values for rat brain [^{125}I]α-bungarotoxin sites show a similar agonist profile, and the identity of homo-oligomeric α7 receptors with native α-bungarotoxin-sensitive neuronal nicotinic receptors is discussed.

Neuronal nicotinic receptor; Nicotinic agonist; (+)-Anatoxin-a; Nicotine; α-Bungarotoxin; *Xenopus* oocyte

1. INTRODUCTION

The α7 nicotinic acetylcholine receptor (nAChR) subunit is unique among vertebrate nAChR subunits characterised so far in its ability to form robust homo-oligomeric channels when expressed in *Xenopus* oocytes [1,2]. The expressed α7 channels respond to acetylcholine (ACh) and nicotine, desensitise very rapidly and are sensitive to α-bungarotoxin (αBgt), curare and dihydro-β-erythroidine [1,3]. Antibodies raised against bacterially-expressed α7 gene product indicate that at least 90% of αBgt-binding proteins in the chick brain contain this subunit [4,5]. Until the cloning and characterisation of the α7 cDNA, the relationship between brain αBgt binding sites and nAChR was ambiguous [6]: although αBgt binding sites have a clear nicotinic profile in binding assays [7], the failure of αBgt to antagonise the majority of centrally-mediated nicotinic responses raised questions about the function of these proteins. More recent studies on autonomic neurons [8,9] have revealed that αBgt-sensitive nAChR are functional but their activity is eclipsed by the dominant nAChR subtype mediating conventional synaptic transmission. In the CNS, it is not known if αBgt-sensitive nAChR perform the same functions as in autonomic neurons. However, αBgt-sensitive channels have been demonstrated by patch-clamp analysis of cultured hippocampal neurons [10,11,12]. These channels show the rapid desensitisation characteristic of α7 nAChR.

Thus there is good evidence that α7 subunits contribute to neuronal αBgt-sensitive nAChR. What is not known is whether other subunits are present in the native protein. Protein chemistry has suggested between 1 and 4 subunits in the αBgt-binding protein [13,14] and antibody studies have indicated that some 20% of α7-containing nAChR in chick brain also contain the related α8 subunit [5]. One approach to the comparison of α7 and native αBgt binding sites is to compare their pharmacological specificities and sensitivities. Semi-rigid agonists, stereoisomers and structural analogues can be particularly discriminating. Here we have examined the effects of seven agonists on α7 nAChR expressed in *Xenopus* oocytes, for comparison with published binding data for brain membranes.

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Abbreviations: nAChR, nicotinic acetylcholine receptor; αBgt, α-bungarotoxin; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; AMP MeI, 1-acetyl-4-methylpiperazine methiodide.

2. EXPERIMENTAL

Xenopus oocytes were injected with 2 ng of α7 cDNA [1]. Electrophysiological recording was performed 2–5 days later using a conventional dual electrode voltage clamp. Cells were clamped at −70 mV and perfused (10 ml/min) with modified Barth's solution (MBS: NaCl 88 mM, KCl 1 mM, HEPES 10 mM, $MgSO_4$ 0.82 mM $Ca(NO_3)_2$ 0.33 mM, $CaCl_2$ 0.91 mM, $NaHCO_3$ 2.4 mM, pH 7.5) containing 0.5 μM atropine. Agonists were applied in perfusion as 3 s pulses. Data were stored on a digital-to-analogue DAT converter and processed using the acquisition and analysis programs AQ and PAT2L [15] and Sigmaplot version 4.1. Dose–response curves were fitted to the non-linear Hill equation: $y = 1/(1+(EC_{50}/x)^{n_H})$, where x = agonist concentration and n_H = Hill number.

Materials. (−)-Nicotine base, ACh-HCl and cytisine were from Sigma Chemical Co., Poole Dorset, UK, DMPP I was from Aldrich, Gillingham, Dorset, and (+)-nicotine hydrogen tartrate was from BDH,

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Poole, Dorset. (+)-Anatoxin-a was provided by Dr. E.X. Albuquerque and AMP MeI was provided by Dr. I. Stolerman. Stock solutions (10 mM) of agonists, except AMP MeI, were made up in MBS and adjusted to pH 7.5 if necessary. Because of the low solubility of AMP MeI, a stock solution (50 mM) was prepared in DMSO. Aliquots were stored at −20°C, thawed on the day of use and diluted in MBS containing 0.5 μM atropine. Control samples of diluted DMSO (no agonist present) were used to confirm that DMSO itself had no effect on oocytes expressing α7.

3. RESULTS

Nuclear injection of α7 cDNA into *Xenopus* oocytes resulted in responses to nicotinic agonists when tested 2–5 days later. Large currents, typically 0.2–2 μA, were recorded (Fig. 1), and these displayed characteristic fast onset and rapid desensitisation at higher agonist concentrations [1]. Currents evoked by (−)-nicotine were completely blocked by 10 nM α-cobratoxin with no recovery following 30 min of washing, whereas 10 nM methyllycaconitine produced a complete blockade that was slowly reversible. Several agonists were investigated for their abilities to activate nicotinic currents (Fig. 1): a range of agonist concentrations was tested on a single oocyte and dose–response curves of the peak currents averaged from several such experiments. (+)-Anatoxin-a was clearly the most potent agonist, with an EC_{50} value of 0.58 μM, whereas ACh (EC_{50} = 320 μM) was the least potent (Table I). Intermediate potencies were observed for cytisine (5.6 μM), (−)-nicotine (24 μM), (+)-nicotine (45 μM), and DMPP (64 μM). Hill slopes were greater than one (Table I), consistent with the binding of more than one agonist molecule for activation; (+)-anatoxin-a and (+)-nicotine produced steeper curves than the other agonists.

When agonist dose–response data were normalised with respect to (−)-nicotine (Fig. 1), it was clear that DMPP elicited much smaller responses than (−)-nicotine despite having comparable potency. A similar effect on α7 nAChR was noted by Bertrand et al. [3]. To explore this further, we examined the structurally related compound 1-acetyl-4-methylpiperazine methiodide (AMP MeI) [16]. The dose–response curve for AMP MeI (Fig. 1) gave an EC_{50} of 170 μM (Table I) and the maximum response was 70% of the maximum

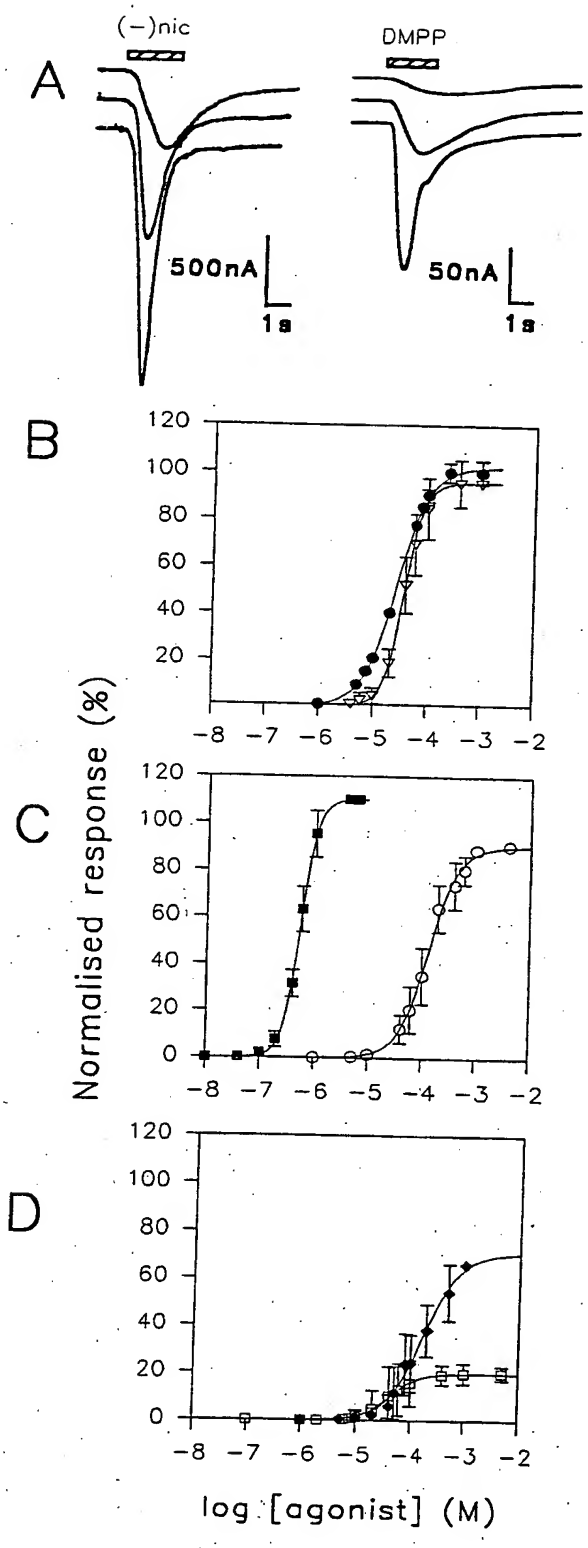

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Fig. 1. Dose–response data for agonists activating α7 nAChR. (A) Superimposed inward currents recorded from two oocytes, each exposed to 3 sec pulses of increasing concentrations of agonist. Ten minute intervals were given between each agonist pulse to allow full recovery from desensitisation, V_H = −70 mV. Left: (−)-nicotine 5×10^{-6}, 5×10^{-5}, 2×10^{-4} M; Right: DMPP 1×10^{-6}, 3×10^{-5}, 1×10^{-3}M. (B,C,D) Dose–response curves compiled from data from several oocytes, and normalised against (−)-nicotine (2.5×10^{-4} M) applied to the same oocyte. Symbols represent the mean data points with S.E.M. indicated by the vertical bars. Lines represent the theoretical dose–response curve fitted to the data points using the non-linear Hill equation (see section 2). (B) ●-●, (−)-nicotine; ▽-▽, (+)-nicotine; (C) ■-■, (+)-anatoxin-a; ○-○, ACh; (D) ◆-◆, AMP MeI; □-□, DMPP.

Table I

Potencies of nicotinic agonists at reconstituted chick α7 nAChR; comparison with ligand binding data for rat brain

Agonist	EC_{50} (M) α7	n_H	Rel. potency (EC_{50})	K_i (M) αBgt binding to rat brain	Rel. potency (K_i)	EC_{50}/K_i
(+)-Anatoxin-a	$5.8 \pm 0.9 \times 10^{-7}$ (5)	2.6 ± 0.3	41	9.1×10^{-8} [a]	97	6.4
Cytisine	$5.6 \pm 1.3 \times 10^{-6}$ (7)	1.9 ± 0.2	23	1.1×10^{-6} [b]	8	5.1
(−)-Nicotine	$2.4 \pm 0.7 \times 10^{-5}$ (4)	1.4 ± 0.1	1	8.9×10^{-6} [c]	1	2.7
(+)-Nicotine	$4.5 \pm 1.0 \times 10^{-5}$ (3)	2.5 ± 0.7	0.53	5.2×10^{-5} [c]	0.17	0.8
DMPP	$6.4 \pm 2.7 \times 10^{-5}$ (7)	2.0 ± 0.2	0.37	7.6×10^{-6} [d]	1.2	8.4
AMP MeI	$1.7 \pm 0.3 \times 10^{-4}$ (5)	1.3 ± 0.1	0.14	3.7×10^{-5} [d]	0.24	4.6
ACh	$3.2 \pm 1.5 \times 10^{-4}$ (5)	1.8 ± 0.3	0.07	1.1×10^{-5} [c]	0.8	29.1

Data from: [a]ref. [7]; [b]ref. [21]; [c]ref. [18]; [d]ref. [16]; [e]unpublished.

response to (−)-nicotine. The efficacies of agonists were compared by applying maximally effective concentrations of each one to the same oocyte in succession (Fig. 2). This confirmed the relative efficacies indicated by the normalised dose–response curves. In particular, DMPP responses were 20% of those of (−)-nicotine whereas AMP MeI gave 66% of the (−)-nicotine response. The plateau level of the AMP MeI dose–response curve (and hence the concentration used in Fig. 2) may be underestimated by the curve fit (Fig. 1) in the absence of data points for higher concentrations of the drug. Such values could not be obtained due to the limited availability and low solubility of AMP MeI. Efficacy comparisons were also carried out with EC_{50} concentrations of the agonists. These experiments confirmed that AMP MeI is at least three times more efficacious than DMPP.

Current–voltage relationships were determined for each agonist and the results are depicted in Fig. 3. The seven agonists behave similarly, responses decreasing as the membrane potential is stepped from −100 to −40

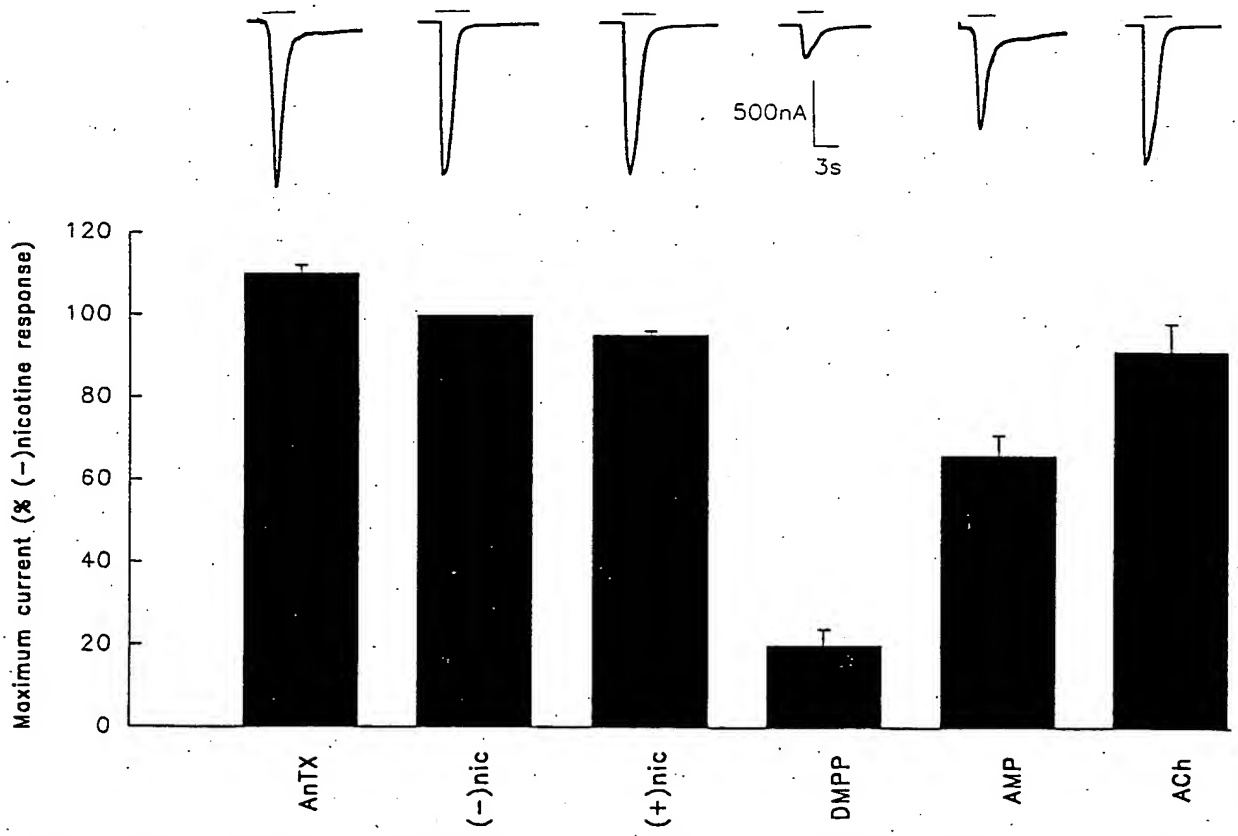

Fig. 2. Efficacy of agonists compared at maximally effective concentrations. Six agonists were tested sequentially on the same oocyte at maximally effective agonist concentrations: (+)-anatoxin-a (4 μM); (−)-nicotine (250 μM); (+)-nicotine (300 μM); DMPP (1 mM); AMP MeI (1 mM) and ACh (4 mM). Current responses were normalised with respect to currents evoked by 250 μM (−)-nicotine. Oocytes were clamped at −70 mV; 12 min intervals were allowed between agonist applications. Values are the mean ± S.E.M. from 5 individual oocytes. Insert: representative current traces.

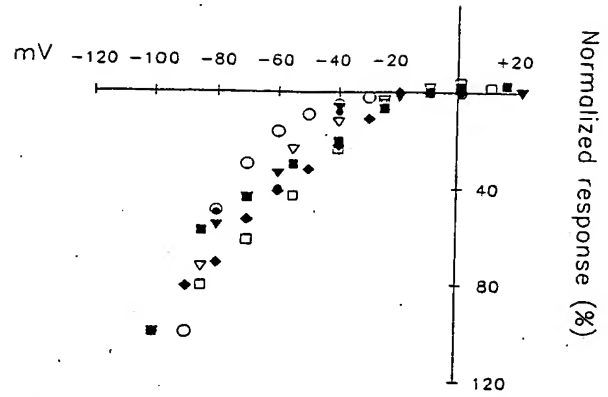

Fig. 3. Current–voltage relationships for six agonists. Applications (3 s) of an agonist were given at 12 min intervals while stepping the holding potential from −100 mV to +20 mV. Current responses were normalised with respect to the response observed at −100 mV. Agonist concentrations approximated to their $EC_{[50]}$ concentrations: ■-■, (+)-anatoxin-a 5×10^{-7} M; ▼-▼, cytisine 5×10^{-6} M; ●-●, (−)-nicotine 2.5×10^{-5} M; ▲-▲, (+)-nicotine 4×10^{-5} M; ▲-▲, DMPP 6×10^{-5} M; ◆-◆, AMP MeI 1×10^{-4} M; ○-○, ACh 2×10^{-4} M.

mV, followed by marked inward rectification at more positive potentials.

4. DISCUSSION

These results extend the quantitative agonist profile of the α7 nAChR. EC_{50} values for (−)-nicotine and ACh are in good agreement with those previously described [3] but cytisine was more potent in the present study. In addition, (+)-anatoxin-a is shown to be the most potent agonist, as it is at other nAChR [17], while (+)-nicotine is two-fold weaker than the naturally occurring enantiomer. Such slight stereoselectivity in favour of (−)-nicotine is a property of αBgt-sensitive sites in *Torpedo* and brain [18], and is in marked contrast to αBgt-insensitive nAChR which show a 100-fold preference for (−)-nicotine [19]. Interestingly, DMPP was a relatively potent agonist at α7 nAChR but displayed only 20% of the efficacy of (−)-nicotine. Bertrand et al. [3] reported that DMPP elicited no significant currents in oocytes expressing α7. Current responses were somewhat larger in the present study and this may have enabled the detection of DMPP-evoked responses. An α7 subunit recently cloned from rat brain [2] also showed anomalous dose-dependency for DMPP, and raised the suggestion that it might have channel blocking activity. Visual inspection of (−)-nicotine and DMPP-evoked current traces (Fig. 1) shows no obvious differences between these two agonists in the rates of activation or desensitisation, but the fast desensitisation shown by α7 nAChR and the limits of resolution imposed by the whole cell recording technique make it difficult to discern the mode of action of DMPP. Analysis is further complicated by the contribution to the α7 responses of a secondary Ca^{2+}-activated chloride current [2]. Single channel analysis will be necessary to determine the precise mechanism that accounts for the small responses observed with DMPP. The structurally related compound AMP MeI was at least 3 times more efficacious than DMPP and may be a fully effective agonist. Thus the common piperazine group is insufficient to explain the results observed with DMPP.

In Table I, the EC_{50} values for agonists are compared with their K_i values for binding to [^{125}I]αBgt sites in rat brain membranes, previously determined in this laboratory. A similar rank order is observed in the two series, but K_i values are approximately five-fold lower than EC_{50} values. However, concordance is not expected as equilibrium binding assays are likely to reflect binding to the desensitised state of the nAChR, and this is associated with higher affinity [20]. The agonists showing greatest deviation from the general relationship between EC_{50} and K_i are (+)-nicotine, which is rather more potent than predicted by the binding data, and ACh, which is less potent than expected. These discrepancies might arise from species differences between chick and rat, or may reflect the absence of additional subunits in the reconstituted nAChR compared to the native receptor. However, αBgt-sensitive nAChR characterised in rat hippocampal neurons (Type IA) [11] have a sensitivity to ACh comparable to that of α7, and the rank order of agonist potencies is similar. Nevertheless an important difference between the native and reconstituted nAChR is that DMPP is as efficacious as ACh at nAChR in hippocampal cells [11]. Secondly, current–voltage relationships for agonists in these neurons may differ from those of α7 nAChR: rectification of α7 currents at about −30 mV (Fig. 3) agrees well with the data of Couturier et al. [1], whereas αBgt-sensitive nAChR in hippocampal neurons have a slightly positive reversal potential and may [10,12] or may not [11] show rectification. Additional subunits in the native protein could account for these differences. Co-expression studies are now required to examine these more complex properties of nicotinic currents, to determine if they are influenced by structural subunits.

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Stable expression and pharmacological properties of the human α_7 nicotinic acetylcholine receptor

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Abstract

The α_7 neuronal nicotinic acetylcholine receptor subtype forms a Ca^{2+}-permeable homooligomeric ion channel sensitive to α-bungarotoxin in *Xenopus* oocytes. In this study, we have stably and functionally expressed the human α_7 cDNA in a mammalian cell line, HEK-293 and examined its pharmacologic properties. [^{125}I]α-Bungarotoxin bound to transfected cells with a K_d value of 0.7 nM and a B_{max} value of 973 pmol/mg protein. No specific binding was detected in untransfected cells. Specific binding could be displaced by unlabeled α-bungarotoxin (K_i = 0.5 nM) and an excellent correlation was observed between binding affinities of a series of nicotinic cholinergic ligands in transfected cells and those in the human neuroblastoma IMR-32 cell line. Additionally, cell surface expression of α_7 receptors was detected by fluorescein isothiocyanate-conjugated α-bungarotoxin in transfected cells. Whole cell currents sensitive to blockade by α-bungarotoxin, and with fast kinetics of activation and inactivation, were recorded from transfected cells upon rapid application of (−)-nicotine or acetylcholine with EC_{50} values of 49 μM and 155 μM respectively. We conclude that the human α_7 subunit when expressed alone can form functional ion channels and that the stably transfected HEK-293 cell line serves as a unique system for studying human α_7 nicotinic receptor function and regulation, and for examining ligand interactions.

Keywords: α_7 Nicotinic acetylcholine receptor subunit; [^{125}I]α-bungarotoxin; HEK-293 (Human embryonic kidney 293 cell); Ion channel, ligand-gated; Cholinergic pharmacology

1. Introduction

A diversity of nicotinic acetylcholine receptor subunits have been identified in the central nervous system in recent years (for reviews see Sargent, 1993; Arneric et al., 1995). To date, 11 gene products (α_2-α_9;β_2-β_4) have been isolated from avian, rodent or human tissues (Sargent, 1993; Elgoyhen et al., 1994). Unlike the α_2-α_6 subunits which require the presence of a β subunit to form a functional receptor (Leutje and Patrick, 1991), the α_7-α_9 subunits are capable of forming functional ion channels when expressed as homooligomers in *Xenopus* oocytes (Couturier et al., 1990; Bertrand et al., 1992; Séguéla et al., 1993; Elgoyhen et al., 1994). In the brain, the predominant classes of nicotinic receptors include those labeled with high affinity by (−)-[^{3}H]nicotine and [^{3}H]acetylcholine corresponding to the $\alpha_4\beta_2$ subunit combination (Whiting et al., 1991; Flores et al., 1992), those labeled with high affinity by [^{125}I]α-bungarotoxin (Clarke et al., 1985) and those labeled with high affinity by [^{125}I]κ-bungarotoxin (Schulz et al., 1991). The distribution of the α_7 gene transcript in rodent brain overlaps the pattern of [^{125}I]α-bungarotoxin binding sites (Séguéla et al., 1993; Clarke et al., 1985) suggesting that the α_7 subunit comprises most, if not all, of the high affinity [^{125}I]α-bungarotoxin-sensitive nicotinic acetylcholine receptor subtype.

The functional role of the putative homooligomeric α_7 subunit remains to be fully defined. Pharmacological and electrophysiological characterization of the α_7 subunit

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expressed in *Xenopus* oocytes indicate that this receptor desensitizes more rapidly than other nicotinic receptors, is highly sensitive to α-bungarotoxin (Couturier et al., 1990) and displays a permeability to Ca^{2+} ions that is significantly greater than those observed for other ligand-gated ion channels including the NMDA subtype of glutamate receptors (Couturier et al., 1990; Bertrand et al., 1992). The marked permeability to Ca^{2+} is suggestive of a possible role for this subtype in excitotoxic processes. A possible role for α_7 nicotinic acetylcholine receptors in neuroprotection/neurodegeneration is suggested by recent findings that levels of neurotrophic factors can be modulated by α-bungarotoxin (Freedman et al., 1993) and that ligands with functional selectivity towards this subtype are neuroprotective in vitro and in vivo (Meyer et al., 1994; De Fiebre et al., 1995). In addition, α_7 gene transcripts are found in relatively high abundance in areas of the rodent brain such as the hippocampus, limbic cortex and thalamus, i.e., regions involved in cognitive function, emotional behavior and states of vigilance (Séguéla et al., 1993). A role for α_7 subtype in schizophrenia is suggested by studies demonstrating that the levels of α_7 transcript are substantially reduced in schizophrenic patients and that acute administration of (−)-nicotine can restore the ability of schizophrenics to gate sensory information (Adler et al., 1992).

We and others have recently cloned the human α_7 nicotinic acetylcholine receptor subunit which when transiently expressed as a functional homomeric ion channel in *Xenopus* oocytes has similar, yet discretely different pharmacological properties when compared to its rat and chick homologues (Doucette-Stamm et al., 1993; Peng et al., 1994). Application of agonists such as (−)-nicotine can elicit rapidly activating and inactivating, inwardly rectifying cation currents in oocytes expressing the α_7 subunit. This current, largely carried by Ca^{2+} ions triggers secondary activation of a large endogenous Ca^{2+}-dependent Cl^- conductance in the oocyte, the latter leading to contamination of agonist-induced responses. The human α_7 nicotinic acetylcholine receptor subunit stably expressed in a mammalian cell line is likely to have biophysical and pharmacological properties more closely resembling those of native receptors and would provide unique opportunities for studying this enigmatic subtype. Recently, Puchacz et al. (1994) described the overexpression of rat α_7 cDNA in a human neuroblastoma cell line, SH-SY5Y. However, studies employing this cell line may be complicated by the presence of endogenously expressed nicotinic receptor subunits, including α_7, in these cells (Peng et al., 1994; Puchacz et al., 1994).

Our studies present the first functional and pharmacologic evaluation of the human α_7 nicotinic receptor subtype stably expressed in a human cell line devoid of endogenous known neuronal nicotinic receptor subunits. The expressed recombinant protein displays receptor binding and pharmacologic properties consistent with those of native α-bungarotoxin receptors and forms functional ion channels that evoke rapidly desensitizing inward currents in response to agonists such as acetylcholine and (−)-nicotine, effects that can be completely blocked by α-bungarotoxin.

2. Materials and methods

2.1. Materials

[^{125}I]α-Bungarotoxin (110–120 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Unlabeled α-bungarotoxin, fluorescein isothiocyanate-labeled α-bungarotoxin, (−)-nicotine bitartrate, (+)-nicotine di-p-toluoyltartrate, acetylcholine chloride, (−)-cytisine, carbachol chloride, (−)-lobeline hydrochloride and atropine methylnitrate were purchased from Sigma Chemical (St. Louis, MO). Methyllycaconitine citrate, (±)-epibatidine dihydrochloride, mecamylamine hydrochloride, dihydro-β-erythroidine hydrobromide, (+)-anatoxin-A hydrochloride and 1,1-dimethyl-4-phenylpiperazinium iodide was purchased from Research Biochemicals International (Natick, MA). The anabaseine-derivatives, 3-(4)-dimethyl-aminocinnamylidine anabaseine hydrochloride and 3-(2,4)-dimethoxybenzylidine anabaseine hydrochloride were synthesized at Abbott Laboratories (Abbott Park, IL). Cell culture media, transfection reagents, fetal bovine serum, geneticin and other antibiotics were obtained from Life Technologies (Grand Island, NY).

2.2. Cell culture

Human embryonic kidney 293 cells and human neuroblastoma IMR-32 cells (American Type Tissue Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3. Molecular biology and transfection

The human α_7 nicotinic receptor cDNA cloned by Doucette-Stamm et al. (1993) was used. The entire coding sequence was excised from the plasmid pBluescript with *Xho*I, the over hang blunt ended with Klenow polymerase, linked with *Bst*XI adapters and ligated into the *Bst*XI site of the expression vector pRcCMV (Invitrogen, San Diego, CA) containing the constitutive human cytomegalovirus promoter to obtain the plasmid pRcCMVα_7. HEK 293 Cells were transfected by lipofectamine™ as described (Felgner, 1991) with some modifications. Briefly, DNA (1.5 μg) was mixed with 20 μg lipofectamine and used to transfect 10^6 cells, previously grown to 50-60% confl-

ence. Forty-eight hours post-transfection cells were split 1:20 and the antibiotic geneticin (0.5 mg/ml) was added to the media to select for resistant clones. After 2 weeks of growth antibiotic-resistant clones were picked at random with cloning cylinders and further propagated in media containing 0.25 mg/ml geneticin. Stably transfected cells, when confluent, were individually analyzed for the binding of [^{125}I]α-bungarotoxin.

2.4. RNA analysis

Total cellular RNA was isolated from untransfected and from stably transfected cells and subjected to reverse-transcription polymerase chain reaction analysis as described previously (Giordano et al., 1992). Reactions were performed using specific primers for the human α_7 (forward nucleotides 346-365, 5′-CGCCACATTCCACACTAAC-3′; reverse complement of nucleotides 602-583, 5′-ACCTTTCACTCCTCTTGCC-3′) and other neuronal nicotinic acetylcholine receptor subunits including α_3, α_4, β_2, β_3 and β_4. Reactions were carried out for 24, 32 and 40 cycles and products visualized by gel electrophoresis. Control reactions were performed in parallel using primers for cyclophilin, an ubiquitous gene, to ensure that the RNA isolation and amplification reactions were successful. As a negative control, reactions were performed using RNA samples subject to first strand cDNA synthesis lacking reverse transcriptase.

2.5. Membrane preparation and ligand binding

Confluent cells were rinsed with ice-cold binding buffer (composition, mM: KH_2PO_4, 50; EDTA, 1 and phenylmethylsulfonyl fluoride, 0.1; pH 7.4 at 25°C), mechanically disaggregated and homogenized using a polytron for 10 s. The homogenate was centrifuged at $45\,000 \times g$ for 30 min at 4°C and the pellet resuspended in ice-cold buffer at a concentration of 50-80 μg protein. Rat cerebral cortex membranes were prepared as described by Enna and Snyder (1975) with minor modifications. Briefly, cerebral cortex were homogenized in 15 volumes of 0.32 M sucrose and centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged at $20\,000 \times g$ for 20 min, the pellets homogenized and centrifuged again at $8000 \times g$ for 20 min. The supernatant was then centrifuged at $40\,000 \times g$ for 20 min and membrane pellet resuspended for use in binding assays.

Membranes were incubated in a final volume of 250 μl with 1 nM radioligand for 120 min at 37°C in the presence or absence of unlabeled drugs. Specific binding was defined by the addition of 1 μM unlabeled α-bungarotoxin to a duplicate set of tubes. For saturation studies, increasing concentrations of [^{125}I]α-bungarotoxin (100 pM–4 nM) were employed. Incubations were terminated by rapid vacuum filtration through GF/B glass fiber filters presoaked in 0.5% polyethyleneimine, and filters washed four times with 2.5 ml of ice-cold buffer. Bound radioactivity was quantitated by gamma counting (80% efficiency) and protein determined using bovine serum albumin as the standard (Lowry et al., 1951).

2.6. Fluorescein isothiocyanate-α-bungarotoxin labeling

Both untransfected and transfected cells expressing α_7 subunit, grown on Lab-Tek chamber slides, were rinsed three times with phosphate buffered saline and fixed in methanol. Slides were then blocked with 0.5% bovine serum albumin for 30 min at room temperature and incubated with diluted fluorescein isothiocyanate-conjugated α-bungarotoxin (1:100). After 60 min, the cells were rinsed three times with saline and mounted in Slowfade™ antifade reagent (Molecular Probes, Eugene, OR). Photomicrographs were taken with a Nikon microscope (Nikon Optiphot UFX-IIA).

2.7. Electrophysiology

Electrophysiological recordings were carried out 1-4 days after plating the cells into 35 mm plastic Petri dishes to allow high power microscopic examination on an inverted Zeiss microscope (ICM 405). Cells were rinsed three times in fresh extracellular medium (composition, mM: NaCl, 120; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 2; D-glucose, 25; Hepes, 10; pH adjusted to 7.4 with NaOH) before recording and continuously superfused with this medium between drug applications. Atropine (1 μM) was added to bath solutions to prevent activation of endogenous muscarinic receptors. Drugs were delivered in the millisecond range by means of a fast, multibarrel, puffer technique (Hu, Maury, Buisson and Bertrand, manuscript in preparation). Whole cell patch clamp recordings were done using standard techniques as described (Hamill et al., 1981). Borosilicate electrodes (with resistance ranging from 2 to 8 MΩ) fashioned on a BB-CH-PC pipette puller (Mecanex, Switzerland), filled with intracellular solution (Composition, mM: KF, 120; KCl, 20; NaCl, 5; $MgCl_2$, 2; Hepes, 10 and EGTA, 0.5; pH adjusted to 7.4 with KOH) were used for recording (Axopatch 200A amplifier, Axon Instruments, Foster City, CA).

2.8. Data and statistical analysis

The binding parameters (K_d and B_{max}) of [^{125}I]α-bungarotoxin (Scatchard, 1959) were determined from analysis of saturation binding isotherm using a nonlinear curve fitting program (LIGAND; Munson and Rodbard, 1980). The K_i values for unlabeled drugs were calculated from the concentration dependence of inhibition using the equation, $K_i = IC_{50}/1 + [[^{125}I]\alpha\text{-bungarotoxin}/K_d]$ (Cheng and Prusoff, 1973). The correlation coefficient of K_i values were determined by linear regression analysis. In electrophysiologic experiments, data was filtered at 2 kHz, acquired at 5 kHz and analyzed using a personal computer equipped with an A–D converter (ATMIO-16D,

National Instruments, USA) and the DATAC package (Bertrand and Bader, 1986). All comparisons were made using unpaired Student's t-test. $P < 0.05$ was considered statistically significant. Values are expressed as means ± S.E.M.

3. Results

3.1. RNA analysis and radioligand binding properties of cells stably transfected with human α_7 cDNA

Colonies of the selected stable integrants were initially analyzed for α_7 receptor expression by examining the binding of 1 nM [^{125}I]α-bungarotoxin. Untransfected cells or cells transfected with the same expression plasmid but containing the open reading frame of the human β_2 nicotinic receptor cDNA showed no specific [^{125}I]α-bungarotoxin binding. From 20 transfected cell clones isolated after selection, cell lines K21, K28, K32, K38 and K41 showed significant (i.e., > 100 fmol/mg protein) specific [^{125}I]α-bungarotoxin binding. The K28 cell line was chosen for further characterization as it displayed the highest density of binding sites. Reverse transcription-polymerase chain reaction analysis of total RNA isolated from the transfected cell line (clone K28) gave a 256 base-pair fragment as expected for the α_7 cDNA at cycles as low as 24. Under similar conditions, no endogenous α_7

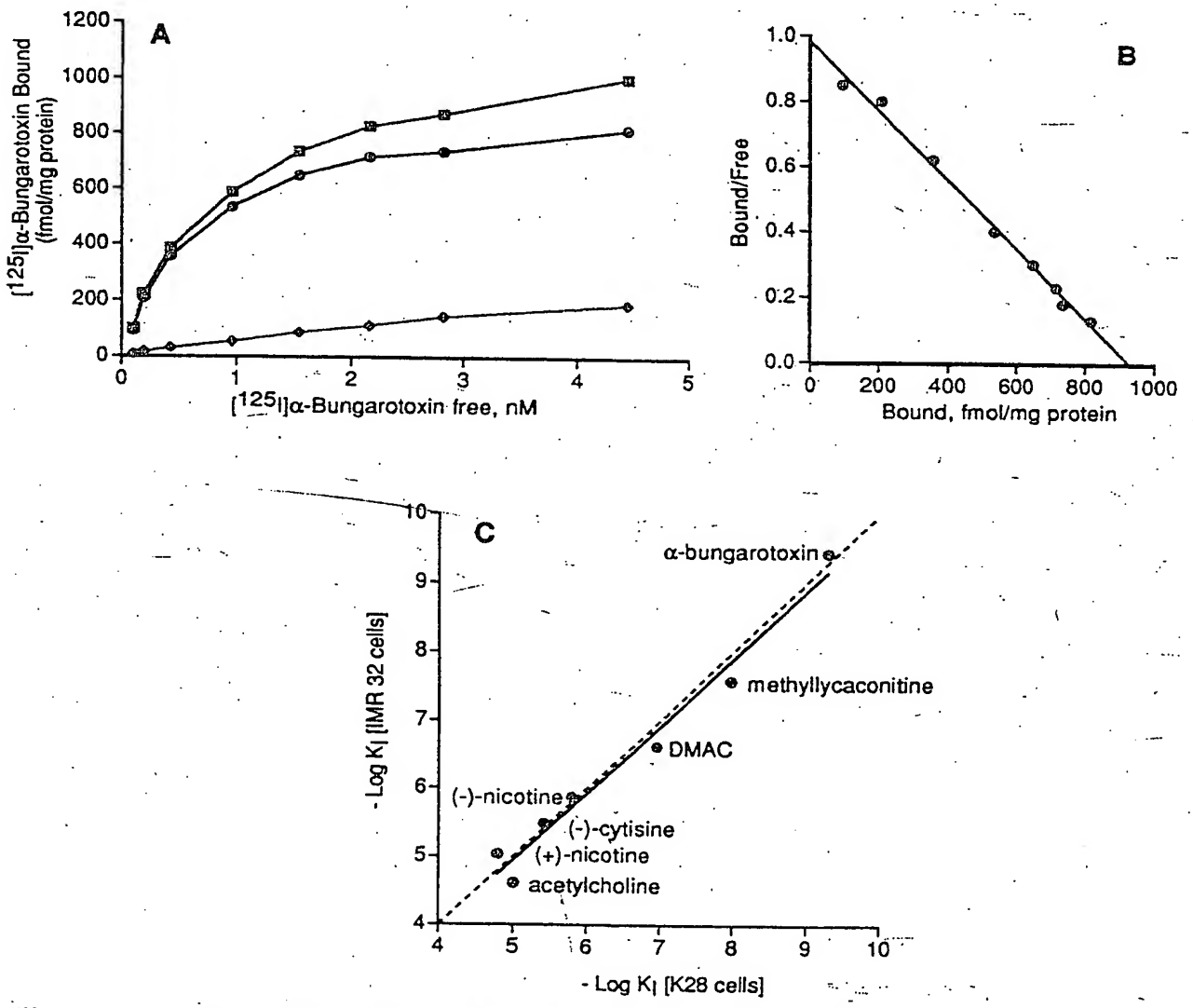

Fig. 1. [^{125}I]α-Bungarotoxin binding properties in K28 cell line stably expressing human α_7 nicotinic acetylcholine receptors (A) Saturation analysis of the binding of [^{125}I]α-bungarotoxin to membranes prepared from K28 cells. Shown are total (squares), specific (circles) and nonspecific (diamonds) binding. (B) Scatchard (Scatchard, 1959) representation of the specific binding data (B_{max} = 959 fmol/mg protein; K_d = 0.76 nM). Depicted is a representative plot. (C) Correlation between binding affinities in K28 cells stably transfected with human α_7 receptors and those in IMR-32 cells. The dashed line represents 1:1 correlation and the solid line represents linear regression through the points (r value = 0.97). Abbreviation: 3-(4)-dimethylaminocinnamylidine anabaseine (DMAC).

message was detected in untransfected HEK 293 cells although positive controls containing cyclophilin cDNA showed the expected 500 base pair band in both transfected and untransfected cells. Further, untransfected cells did not reveal the expected base-pair fragments corresponding to human α_3, α_4, β_2, β_3 and β_4 subunits at any of the amplification cycles examined indicating no endogenous expression of these known neuronal nicotinic receptor subunits, consistent with observations from other laboratories (Wong et al., 1995). In support of this finding, untransfected cells did not exhibit specific [^{125}I]α-bungarotoxin binding and failed to show nicotinic receptor-mediated functional responses assessed by agonist-evoked $^{86}Rb^+$ flux (data not shown) or ionic currents (see below).

[^{125}I]α-Bungarotoxin bound saturably and with high affinity to the transfected K28 cells stably expressing the human α_7 subunits (Fig. 1A). Within the radioligand concentration range examined, specific binding was to a single set of saturable binding sites with a K_d value of 0.71 ± 0.11 nM and maximal binding density of 973 ± 140 fmol/mg protein (($n = 6$; Fig. 1B). Specific binding defined by 1 μM unlabeled α-bungarotoxin represented 80-95% of the total bound ligand. The levels of expression in K28 cell line is significantly higher than the corresponding values observed in the human neuroblastoma, IMR-32 (180 ± 20 fmol/mg protein; $n = 4$) or in rodent brain (69 ± 12 fmol/mg protein; $n = 3$) while the K_d value for the K28 cell line is in excellent agreement with that found in IMR-32 cells (0.65 ± 0.13 nM; $n = 4$)) or in rodent brain (0.70 ± 0.15 nM; $n = 3$). Importantly, binding properties of K28 cell line have been maintained in culture for > 9 months and cells could be retrieved from frozen storage without loss in activity.

The pharmacology of [^{125}I]α-bungarotoxin binding to K28 cells was examined in the presence of 1 nM [^{125}I]α-bungarotoxin, which is close to the K_d value (0.7 nM). The potent inhibitors of binding besides α-bungarotoxin

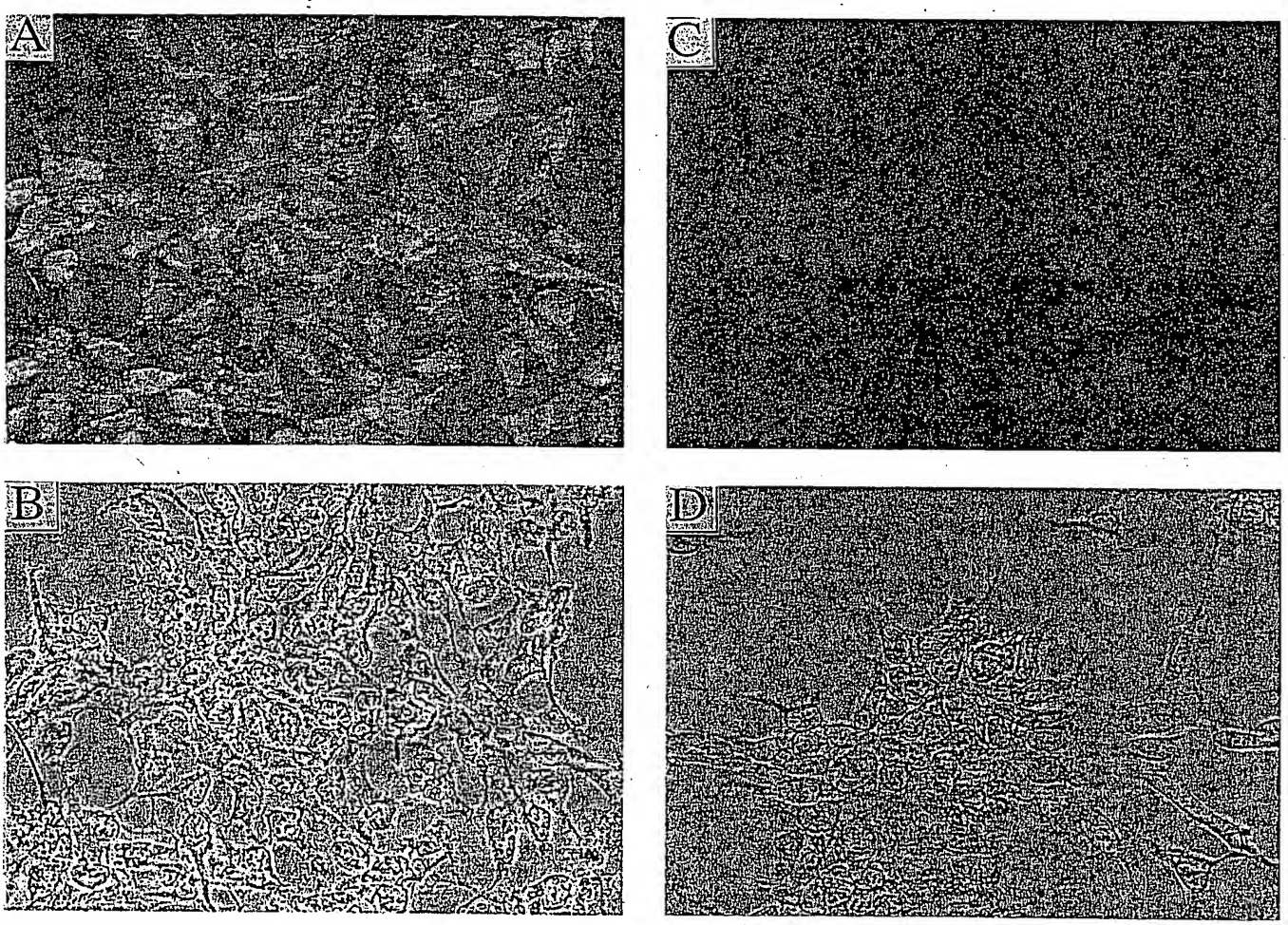

Fig. 2. Visualization of human α_7 nicotinic receptors in K28 cells by α-bungarotoxin. (A) Dark-field photomicrograph showing fluorescence labeling of K28 cells with α-bungarotoxin (B) Phase-contrast micrograph of the cells shown in A. (C) α-Bungarotoxin binding to untransfected cells showing background labeling and (D) Phase-contrast micrograph of the cells shown in C. Fluorescein isothiocyanate-conjugated α-bungarotoxin was employed and cells were cultured on poly-L-lysine-coated dishes (magnification = 1200 ×).

include methyllycaconitine, a selective antagonist for the neuronal α-bungarotoxin-sensitive receptors (Wonnacott et al., 1993), (±)-epibatidine and (+)-anatoxin-A (Table 1). The anabaseine-derivatives, 3-(4)-dimethyl-aminocinnamylidine anabaseine and 3-(2,4)-dimethoxybenzylidine anabaseine belonging to a class of functionally selective α_7 agonists (Meyer et al., 1994; De Fiebre et al., 1995) also inhibited [^{125}I]α-bungarotoxin binding. The relative affinities of the various ligands at the recombinant α_7 subtype was found to be, α-bungarotoxin (0.48 ± 0.07 nM; $n = 4$) > methyllycaconitine > (±)-epibatidine > (+)-anatoxin-A > 3-(4)-dimethylaminocinnamylidine anabaseine > 3-(2,4)-dimethoxybenzylidine anabaseine > anabaseine > (−)-nicotine > acetylcholine (9910 ± 470 nM; $n = 3$). Inhibition of binding by nicotine was stereoselective with the (−)-enantiomer being 10-fold more potent than the (+)-enantiomer. The affinities of these ligands for [^{125}I]α-bungarotoxin binding sites in the human neuroblastoma IMR-32 were similar and a very good correlation (r value = 0.97) was observed between the K_i values in K28 cells and those in IMR-32 cells [Fig. 1C]. Other cholinergic ligands examined were effective only at concentrations much greater than required for interaction at their defined receptor type(s) (Table 1). For example, (−)-cytisine,

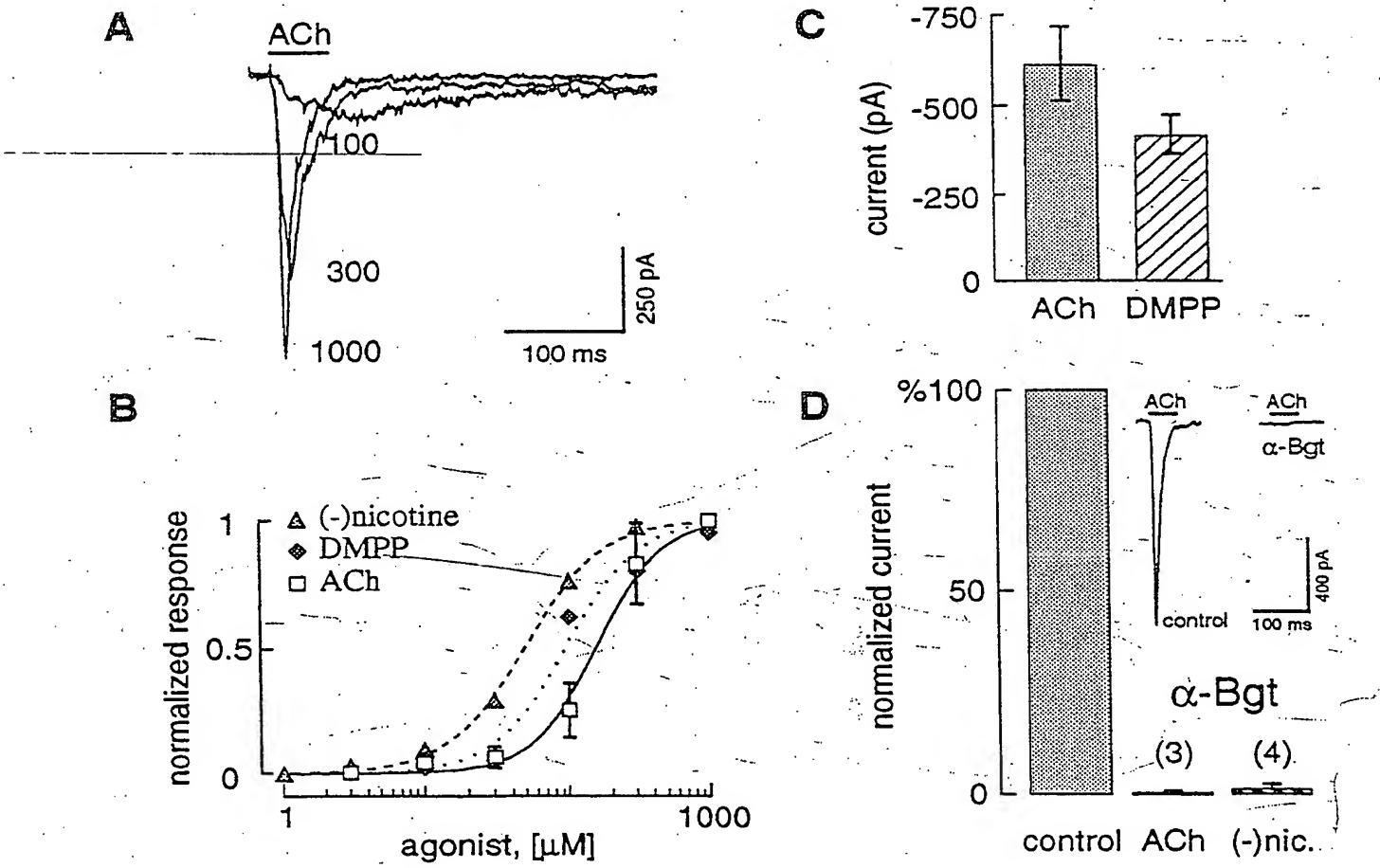

Fig. 3. Functional and physiological properties of the human α_7 nicotinic acetylcholine receptors stably expressed in K28 cells. In all conditions, cells were held at −100 mV and pulses of agonist of 50 ms duration were applied once every 3 s (A) Whole cell currents elicited by increasing acetylcholine concentrations (100, 300 and 1000 μM; the 50 ms application is indicated by the horizontal bar). (B) Dose response curves for acetylcholine (open squares; 14 cells), (−)-nicotine (filled triangles; 13 cells) and 1,1-dimethyl-4-phenylpiperazinium (filled diamond; 12 cells). Currents evoked by short pulses of agonist given from the lowest to higher concentrations were measured, normalized to their maximum value and plotted as a function of the log of the agonist concentration. Continuous (acetylcholine) and dotted (1,1-dimethyl-4-phenylpiperazinium and (−)-nicotine) lines were obtained using the empirical Hill equation. The best fit for acetylcholine was obtained with an EC_{50} value of 155 μM and a Hill coefficient of 1.9, whereas for (−)-nicotine and 1,1-dimethyl-4-phenylpiperazinium the values were, respectively, 49 μM (1.6) and 95 μM (1.7). For clarity, error bars have been omitted for (−)-nicotine and 1,1-dimethyl-4-phenylpiperazinium. (C) At saturating concentrations (1 mM; 50 ms), acetylcholine is more efficacious than 1,1-dimethyl-4-phenylpiperazinium in eliciting fast transient currents. The mean current elicited by 1,1-dimethyl-4-phenylpiperazinium represented only 70.2% of the mean acetylcholine-evoked current ($n = 7$). (D) Acetylcholine-and (−)-nicotine-evoked currents are blocked by α-bungarotoxin. Currents were elicited by applications of 1 mM acetylcholine or 300 μM (−)-nicotine. After 1 min perfusion of α-bungarotoxin, 100 nM, acetylcholine-evoked currents are reduced by 99.6 ± 0.5% ($n = 3$) and (−)nicotine-evoked currents by 98.7 ± 1.3% ($n = 4$). The inset shows a current prior to (control) and after (α-bungarotoxin) 1 min perfusion with α-bungarotoxin (as indicated). Abbreviations: acetylcholine (ACh); 1,1-dimethyl-4-phenyl piperazinium (DMPP); α-bungatoroxin (α-BgT).

Table 1
[^{125}I]α-Bungarotoxin binding affinities of nicotinic receptor ligands in the K28 cell line

Compound	K_i (nM)
α-Bungarotoxin	0.48 ± 0.07
Methyllycaconitine	10.3 ± 3.0
(±)-Epibatidine	20.6 ± 4.0
(+)-Anatoxin-A	63.3 ± 7.3
DMAC	111 ± 10
DMXB	652 ± 7
Anabaseine	759 ± 133
(−)-Nicotine	1610 ± 220
(−)-Cytisine	3883 ± 1050
Acetylcholine	9910 ± 470
(−)-Lobeline	13 100 ± 2500
(+)-Nicotine	16 050 ± 2080
Atropine	16 900 ± 5600
Carbachol	21 500 ± 5500
Dihydro-β-erythroidine	57 900 ± 1700
Mecamylamine	> 100 000

Radioligand binding was performed to K28 cell membranes as described under Materials and methods using [^{125}I]α-bungarotoxin, 1 nM. Values represent mean ± S.E.M of 3 to 4 independent determinations, each performed in duplicate. Hill coefficient (n_H) were close to unity in all cases. Abbreviations: 3-(4)-dimethylaminocinnamylidine anabaseine (DMAC); 3-(2,4)-dimethoxybenzylidine anabaseine (DMXB).

(−)-lobeline and dihydro-β-erythroidine display K_i values of 0.14, 1.4 and 15 nM respectively for the inhibition of high affinity [^{3}H]cytisine binding to the rat cerebral cortex (Anderson and Arneric, 1994) while carbachol displays a K_i value of 9.9 nM for the inhibition of [^{3}H]oxotremorine binding to the rat cortical muscarinic receptors (Anderson and Arneric, 1994).

3.2. Fluorescence labeling of α_7 nicotinic acetylcholine receptors in transfected cells

The expression of the α_7 nicotinic acetylcholine receptors on the cell surface was examined by fluorescein isothiocyanate-conjugated α-bungarotoxin labeling. Greater than 80% of the K28 cells could be labeled by fluorescent α-bungarotoxin with the label appearing as uniformly distributed punctations throughout the cell surface (Fig. 2A). No significant labeling was observed on untransfected HEK-293 cells (Fig. 2C). The labeling on K28 cells was abolished by preincubation with unlabeled α-bungarotoxin (10 nM) indicating specificity in α_7 receptor expression (not shown). Further, a human α_7 receptor specific antibody revealed intense labeling in K28 but not in untransfected cells (Marietta Piattoni-Kaplan et al., manuscript in preparation).

3.3. Functional properties of α_7 nicotinic acetylcholine receptors in transfected cells

The functional properties of the expressed α_7 nicotinic acetylcholine receptors were evaluated using the whole cell patch clamp technique. Inward currents (ranging from 28 to 3068 pA) were elicited by short pulses of saturating agonist concentrations in K28 cells (Fig. 3A). Among the transfected cells examined, we have observed only 3 (out of 75) cells that did not display acetylcholine-or (−)-nicotine-evoked currents. Those 3 cells were challenged the day after subculture and the absence of currents could have resulted from trypsin degradation of membrane-associated receptors. Current amplitudes were also much smaller the day after plating than during the subsequent days. We did not record any acetylcholine-evoked currents in untransfected cells (1 mM acetylcholine, 50 ms; $n = 7$) indicating that the currents elicited in transfected cells resulted from functional α_7 nicotinic acetylcholine receptor expression following stable transfection with human α_7 cDNA. The transfected cells displayed currents characterized by very fast kinetics of activation and inactivation, and with a mean time-to-peak of 6 ± 0.6 ms (100 μM (−)-nicotine, 50 ms; $n = 11$) which is very similar to the value (7 ms) previously described for the fast transient currents recorded from SH-SY5Y cells stably transfected with the rat α_7 cDNA (Puchacz et al., 1994). The inactivation component of the current could be fitted, on a typical cell, with a single exponential function with a time constant of viz., 12, 20 and 250 ms for respective acetylcholine concentrations of 1000, 300 and 100 μM. Half-effective concentrations (EC_{50}, calculated using the empirical Hill equation) for acetylcholine and (−)-nicotine were 155 μM and 49 μM respectively (Fig. 3A and 3B). We also observed that 1,1-dimethyl-4-phenylpiperazinium behaved as an agonist at the human α_7 receptors with an EC_{50} of 95 μM (Fig. 3B). However at saturating concentrations, 1,1-dimethyl-4-phenylpiperazinium is less efficacious than acetylcholine and could elicit only 70% of the mean acetylcholine-evoked currents ($n = 7$; Fig. 3C). These results indicate that 1,1-dimethyl-4-phenylpiperazinium could be a partial agonist and/or behave as an open channel blocker at high concentrations. The acetylcholine-or (−)-nicotine-evoked currents were completely inhibited by 99.6 ± 0.3% ($n = 3$) and 98.7 ± 1.3% ($n = 4$) respectively after one minute superfusion of α-bungarotoxin (100 nM; Fig. 3D). Moreover, methyllycaconitine (1 nM) inhibited (−)-nicotine-evoked currents with partial recovery after washout (data not shown).

4. Discussion

This study reports the stable and functional expression of the human α_7 nicotinic acetylcholine receptor subunit in a somatic cell line. We demonstrate that in the clonal K28 cell line, α_7 subunit cDNA can direct the expression of a functional ion channel that binds [^{125}I]α-bungarotoxin with the appropriate pharmacology, and elicits rapidly desensitizing α-bungarotoxin-sensitive inward currents in response to the classical nicotinic receptor agonists such as

(−)-nicotine and acetylcholine. In contrast to previous studies that necessitated induction for expression of nicotinic receptors (AM4 cells expressing the $\alpha\beta\gamma\delta$ subunits, Sine and Claudio, 1991; M10 cells expressing the $\alpha_4\beta_2$ subunits, Whiting et al., 1991), stable expression of functional human α_7 subunit has been accomplished constitutively in K28 cells.

The pharmacology of the α_7 receptor subtype expressed in K28 cells is consistent with that of the native α-bungarotoxin sensitive receptors expressed in the human neuroblastoma IMR-32 cell line. The observed affinity for α-bungarotoxin is similar to that reported for human α_7 homomers transiently expressed in *Xenopus* oocytes (0.8 nM, Peng et al., 1994) and those reported in human neuroblastoma lines such as SH-SY5Y (1 nM, Peng et al., 1994) or IMR-32 (0.6 nM, this study). (+)-Anatoxin-A and (±)-epibatidine, both agonists at the homomeric α_7 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes (Amar et al., 1993; Sullivan et al., 1994) inhibited [^{125}I]α-bungarotoxin binding with K_i values in the nanomolar range (Table 1). As shown in Fig. 1C, there exists a good correlation between [^{125}I]α-bungarotoxin binding affinities of a series of nicotinic cholinergic ligands in K28 and IMR-32 cells indicating that the expressed α_7 receptors in K28 cells behave very similarly to those present in native tissues. The maximal binding site density in K28 cells is 5-to 15-fold higher than those found in native rodent brain or in neuroblastoma cell lines, but comparable to the levels observed in human SH-SY5Y cells overexpressing the rat α_7 cDNA (Puchacz et al., 1994).

Until recently, detection of functional responses following activation of the α_7 receptors in clonal cell lines or cultured neuronal cells has been limited perhaps due to the rapidly desensitizing nature of the response. However, by rapid application of agonists such as (−)-nicotine and acetylcholine, it has been shown that native α-bungarotoxin binding receptors present in cultured neurons can function as ligand gated cation-permeant channels and can elevate intracellular Ca^{2+} levels (Vijayaraghavan et al., 1992; Zhang et al., 1994). The observation that K28 cells exhibited currents with rapid kinetics of activation and inactivation in response to acetylcholine and other agonists, sensitive to blockade by α-bungarotoxin, suggests that the human α_7 receptor can function as a homomer in this non-neuronal cell line although the possibility that other, as yet unidentified, neuronal nicotinic receptor subunits are expressed in HEK-293 cells cannot be discounted at this point. The EC_{50} values for acetylcholine, (−)-nicotine and 1,1-dimethyl-4-phenylpiperazinium reported in the present study are higher when compared with those recently published for the human α_7 receptors expressed in *Xenopus* oocytes (Peng et al., 1994). This discrepancy could be explained by the following observations. Although the α_7 receptors are highly permeable to Ca^{2+} ions (Séguéla et al., 1993; Bertrand et al., 1993; Sands et al., 1993), the oocytes used in the study of Peng et al. (1994) were recorded in the absence of intracellular Ca^{2+} buffer (containing for example 1,2-bis(2-aminophenoxy)ethane tetraacetic acid, BAPTA). Under such recording conditions, it has been shown that endogenous Ca^{2+}-activated Cl^- currents shift the dose-response curve to the left, i.e., increase the potency (Eiselé et al., 1993). Our patch electrodes contained 0.5 mM EGTA that was enough to prevent activation of Ca^{2+}-activated Cl^- currents. This could explain the differences in the EC_{50} values presented here and those reported by Peng et al. (1994). However, it is noteworthy that the EC_{50} values for acetylcholine and (−)-nicotine observed in our study are in close agreement with the values previously measured for the SH-SY5Y cells transfected with the rat α_7 cDNA (Puchacz et al., 1994). The inactivation time constant of acetylcholine-evoked current in K28 cells is in the range calculated for the α-bungarotoxin-sensitive currents of chick ciliary ganglion neurons (Zhang et al., 1994) and of cultured rat hippocampal neurons (Zorumski et al., 1992; Alkondon and Albuquerque, 1993). Altogether, these results clearly establish that the fast acetylcholine-or (−)-nicotine-evoked transient currents resulted from activation of human α_7 nicotinic receptors stably expressed in K28 cells. In addition, the current-voltage relationship for acetylcholine-evoked currents was found to strongly rectify and this compares well with those described either for the α_7 nicotinic receptors heterologously expressed in *Xenopus* oocytes (Couturier et al., 1990; Séguéla et al., 1993; Peng et al., 1994) or in SH-SY5Y cells transfected with the rat α_7 cDNA (Puchacz et al., 1994).

The physiologic roles of the [^{125}I]α-bungarotoxin-sensitive α_7 nicotinic acetylcholine receptors remain unclear. It has reported roles in neurite growth and synaptic transmission and in the induction of long-term potentiation (Pugh and Berg, 1994; Hunter et al., 1994). The recent availability of functionally selective ligands such as 3-(4)-dimethylaminocinnamylidine anabaseine has provided evidence for a neuroprotective role as well (Meyer et al., 1994; De Fiebre et al., 1995). Additional support for this possibility is provided by the observation that α-bungarotoxin can modulate the levels of neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor in rat hippocampus (Freedman et al., 1993). A possible involvement of the α_7 receptors in epileptic seizures may be indicated by the higher density of [^{125}I]α-bungarotoxin binding sites in a mouse strain with increased sensitivity to nicotine-induced seizures (Miner et al., 1986). Furthermore, there is emerging evidence to link changes in the expression the α_7 receptors with the pathology of schizophrenia (Adler et al., 1992). However, the mechanisms of receptor regulation in these disease states have not been elucidated. The availability of an in vitro model such as the K28 cell line expressing the human α_7 subtype presents an opportunity for investigating the structure, function and regulation of this enigmatic receptor subtype

and for examining ligand interactions. Our study provides the first pharmacological characterization of the human α_7 receptors stably expressed in a human cell line lacking other known neuronal nicotinic receptor subunits. Similar studies with other subunits or subunit combinations will ultimately set the stage for identifying potential therapeutic targets that selectively modulate each of the various nicotinic acetylcholine receptor subtypes.

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CHAPTER 9

Nicotinic acetylcholine receptors on hippocampal neurons: cell compartment-specific expression and modulatory control of channel activity

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Introduction

Neuronal nicotinic receptors (nAChRs) are ACh-gated cationic channels and belong to a superfamily of ligand-gated ion channels (Betz, 1990). The neuronal nAChRs are believed to be penta-oligomers made up of a single type of the agonist-binding α subunit or of a combination of α subunits with structural, β subunits (Anand et al., 1991; Role, 1992; Karlin, 1993; Sargent, 1993). Screening of mRNA libraries from neurons of various brain regions of rats, chicks, and humans resulted in the isolation, identification, and cloning of eight α (α2–α9) and three β (β2–β4) neuronal nAChR subunits (Heinemann et al., 1991; Lindstrom, 1995). According to studies of ectopic expression of a variety of combinations of nAChR subunits, out of the eight α subunits cloned to date, only three (α7, α8, and α9) can form homooligomeric functional neuronal nAChRs; all the other α subunits have to be combined with β subunits to give rise to functional nAChRs (Lindstrom, 1995). Because of the different possible combinations of the various neuronal nAChR subunits, neuronal nAChRs are diverse in their functional and pharmacological properties (Role, 1992; Albuquerque et al., 1995a).

Based on binding studies, neuronal nAChRs were classified into two broad classes: the α-BGT-insensitive nAChRs (which include the nAChRs that bind nicotine with high affinity) and the α-BGT-sensitive nAChRs (Romano and Goldstein, 1980; Lukas, 1984; Clarke et al., 1985). Electrophysiological studies carried out in CNS neurons suggested that various functional nAChR subtypes an be found in a single brain area, and that in some brain areas a single neuron can express more than one subtype of neuronal nAChR (Alkondon and Albuquerque, 1993, 1995; Alkondon et al., 1994). It is less clear whether there is segregation of the distinct subtypes of neuronal nAChRs on the apical and basal dendrites, cell body, and dendritic spines of the neurons, or whether rather than being segregated on these different regions, the various nAChR subtypes would be intermingled on the entire neuronal surface. In addition, it is unknown whether the nAChRs expressed on postsynaptic neurons in the CNS are synaptic or extrasynaptic receptors. However, the great diversity of neuronal nAChRs, in addition to the fast rate of activation/inactivation of some of these receptors in the CNS, still hinders their functional characterization in situ (Albuquerque et al., 1995b).

There is mounting evidence in support of the notion that ACh plays an important role in learning and memory, as well as in the control of

neuronal development, partly by binding to neuronal nAChRs in the CNS. Given that the hippocampus is the brain structure most prominently associated with cognitive functions, our studies have been focused in the characterization of the properties of nAChRs expressed on hippocampal neurons.

Hippocampal neurons either in culture or acutely dissociated respond to nicotinic agonists with at least one of three pharmacologically and kinetically distinct ionic currents, which are referred to as type IA, II, and III currents (Alkondon and Albuquerque, 1993, 1995; Alkondon et al., 1994; Ishihara et al., 1995a). Type IA currents, the predominant responses of hippocampal neurons to nicotinic agonists, are fast-desensitizing currents sensitive to blockade by methyllycaconitine (MLA, 1 nM) or α-bungarotoxin (α-BGT, 10 nM) (Alkondon et al., 1992; Alkondon and Albuquerque, 1993). Type II currents, which can be recorded from 5% of the tested neurons, are slowly desensitizing currents sensitive to blockade by dihydro-β-erythroidine (DHβE) (10 nM) (Alkondon and Albuquerque, 1993, 1995). Type III currents, nicotinic responses recorded from 2.5% of the tested neurons, are slowly desensitizing currents that are sensitive to blockade by mecamylamine (1 μM) (Alkondon and Albuquerque, 1993). About 10% of the hippocampal neurons respond to nicotinic agonists with whole-cell currents that have two components. The fast-decaying component of these currents (which are referred to as type IB currents) has the same pharmacological and biophysical properties as type IA currents, whereas the slowly decaying component of these currents have the same characteristics as type II currents, in this way indicating that a single hippocampal neuron can express more than one nAChR subtype (Alkondon and Albuquerque, 1993, 1994; Alkondon et al., 1994). It is likely that an α7-bearing nAChR gives rise to type IA currents, an $\alpha 4\beta 2$ nAChR gives rise to type II currents, and an $\alpha 3\beta 4$ nAChR gives rise to type III currents (Alkondon and Albuquerque, 1993). Indeed, hippocampal neurons express mRNAs coding for nAChR α7, α4, and β2 subunits (Alkondon et al., 1994), and the proportion of hippocampal neurons binding either nicotine with high affinity or α-BGT corresponds to the proportion of neurons that respond to nicotinic agonists with type II or type IA currents, respectively (Barrantes et al., 1995). Also in agreement with the concept that α7-bearing nAChRs account for the α-BGT-sensitive nicotinic currents recorded from hippocampal neurons was the finding that the α-BGT-sensitive hippocampal nAChRs have a high permeability to Ca^{2+} as does the homomeric α7-based nAChR ectopically expressed in *Xenopus* oocytes (Vijayaraghavan et al., 1992; Séguéla et al., 1993; Castro and Albuquerque, 1995).

Expanding these studies, we now show for the first time that neuronal nAChRs are differentially expressed on the somato-dendritic surface of hippocampal neurons. In addition, we demonstrate that various ions and drugs play a crucial role in modulating the activity of neuronal nAChRs.

Neuronal nAChRs and cholinergic synapses in the hippocampus

Very recently, we have concentrated our efforts on the identification and characterization of the different types of nicotinic responses that can be recorded by focal application of nicotinic agonists to the dendrites and to the soma of hippocampal neurons that are visualized in slices obtained from 13–18-day-old rats or of cultured hippocampal neurons (Alkondon et al., 1995). Using infrared differential interference contrast videomicroscopy (Spruston et al., 1995) and a computerized system of micromanipulators, a drug-delivery pipette having a tip diameter of $<2\,\mu$m can be positioned near the neuronal cell body, the apical or basal dendrites, or the dendritic regions surrounding the spines.(Alkondon et al., 1996). When rapidly and locally applied to the cell body and to various regions of the apical and basal dendrites of hippocampal neurons, either in slices or in cultures, ACh and other nicotinic agonists can activate nicotinic currents (Figs. 1 and 2). These studies demonstrate for the first time that functional nAChRs can be found on the entire surface of hippocampal neurons, including their dendrites and

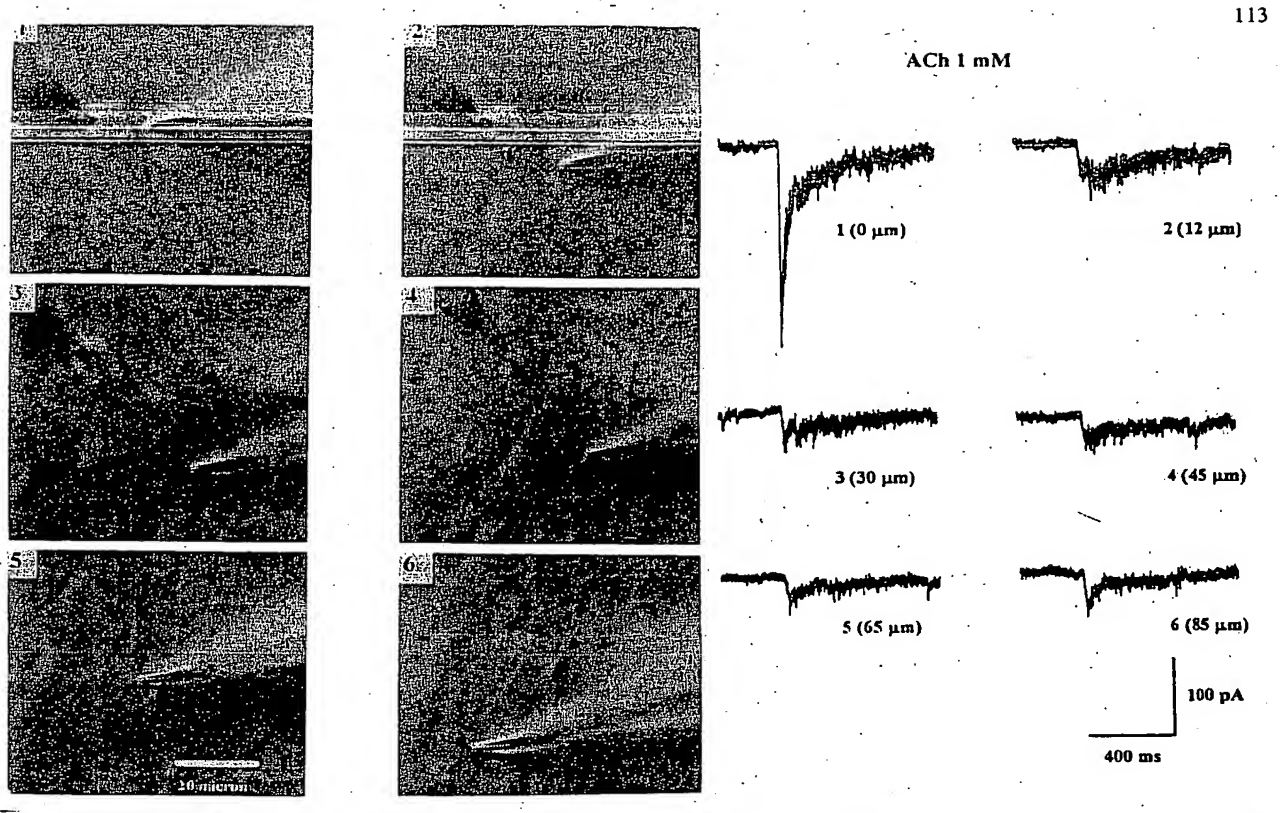

Fig. 1. Infrared images of a cultured hippocampal neuron and samples of the ACh-evoked currents from the same neuron. On the left: set of images of the neuron with the recording pipette on the cell soma and the ACh-delivery pipette positioned near the cell body or at various locations along the apical dendrite. All images have been contrast enhanced using a Hamamatsu Image Processor. On the right: sample recordings of whole-cell currents evoked by a 10-ms pressure application of ACh (1 mM) to the various regions of the neuron. Numbers 1–6 represent the position of the ACh-delivery pipette as shown on the left. The distance of the application pipette relative to the center of the cell body is shown in the parentheses. Despite the cable attenuation of the nicotinic currents, the presence of nAChR on the apical dendrite at distances as far as 85 μm from the cell body is clearly demonstrated. Material and methods: neurons dissociated from the hippocampi of 16–18-day-old fetal rats were cultured on polylysine-precoated coverslips following methods described previously (Alkondon and Albuquerque, 1993). The composition of the external solution used to bathe the neurons and to dilute agonists and test compounds was (in mM): NaCl 165, KCl 5, $CaCl_2$ 2, HEPES 5 and dextrose 10 (pH = 7.3; osmolarity = 340 mOsM). The composition of the internal solution was (in mM): CsCl 60, CsF 60, EGTA 10, HEPES 10 (pH = 7.3; osmolarity = 330 mOsM) and to prevent to a great extent the rundown of the nicotinic currents, phosphocreatine (20 mM), creatine phosphokinase (50 units/ml) and ATP (5 mM) were added to this solution. Whole-cell, patch-clamp experiments were performed on 21–29-day-old-cultured hippocampal neurons. Whole-cell patches from the soma of the hippocampal neurons were made under visual control using infrared differential interference contrast (IR-DIC) videomicroscopy (Spruston et al., 1995), utilizing an infrared filter (RG-9, λ 800 nm, Melles Griot) and a Newvicon camera (C2400-07; Hamamatsu, Hamamatsu City, Japan). The images were gathered using a frame grabber and further enhanced using an Argus-10 image processor (Hamamatsu City, Japan). The patch pipettes were pulled from borosilicate capillary glass and had tip diameters ranging from 2 to 4 μm. The resistance of the patch pipettes, when filled with internal solution, was between 3 and 5 MΩ. Whole-cell currents were induced by fast application of ACh (1 mM) to the neurons using a glass "U"-shaped tube positioned about 100 μm from the cell and recorded using an LM-EPC-7 patch-clamp system (List Electronics, Darmstadt, Germany). The signals were filtered at 3 kHz and directly sampled by a microcomputer using the pCLAMP program (version 6.0, Axon Instruments, Foster City, CA). A robotic system was used to control the movements of the micromanipulators that held the patch pipette and the drug-delivery pipette. The movements of these micromanipulators ranged from 20 nm to 1 cm. Whole-cell currents evoked by application of ACh to the various regions on the surface of the neuron were recorded using an LM-EPC-7 patch-clamp system (List Electronics, Darmstadt, Germany) and according to standard patch-clamp technique (Hamill et al., 1981). The signals were filtered at 3 kHz and directly sampled by a microcomputer using the pCLAMP program (for further details see Alkondon et al., 1995).

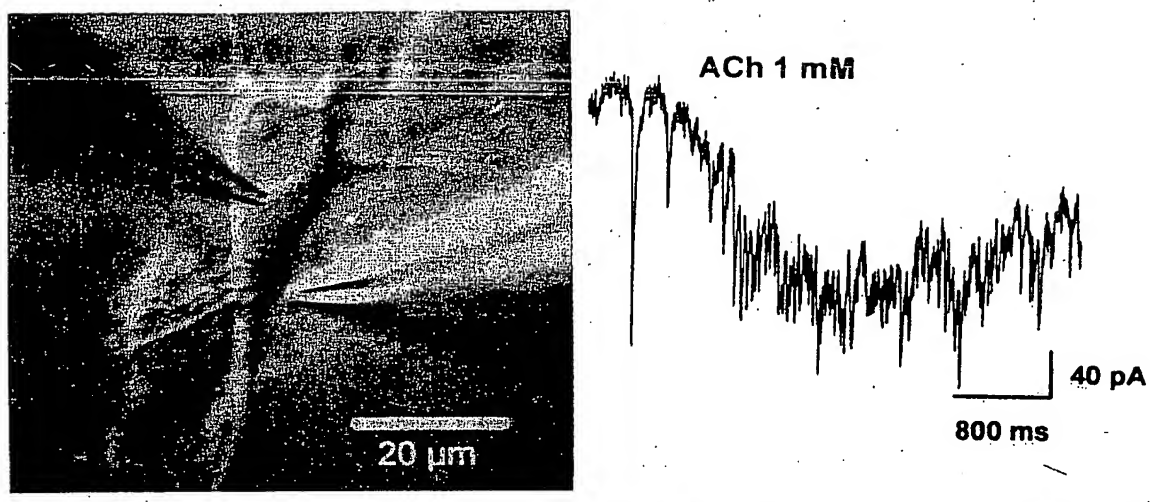

Fig. 2. Infrared image of a pyramidal neuron visualized in the CA1 area of a 200-μm thick hippocampal slice and samples of the nicotinic currents recorded from the same neuron. On the left: image of the neuron with the recording pipette on the cell soma and the ACh-delivery pipette positioned near the basal dendrite. The image was contrast enhanced using a Hamamatsu Image Processor. On the right: sample recordings of the currents evoked by a 10-ms pressure application of ACh (1 mM). Material and methods: hippocampal slices were prepared according to the procedure described elsewhere (Spruston et al., 1995) and all electrophysiological experiments were made according to the procedures described in Fig. 1. For further details see Alkondon et al. (1995).

dendritic regions surrounding the spines. The activity of neuronal nAChRs located in discrete areas of the dendrites could account for some of the changes in synaptic morphology that are intimately associated with cognitive processes (Desmond and Levy, 1986; Markus and Petit, 1987; Markus et al., 1987).

It has been proposed that increases in intracellular Ca^{2+} levels by Ca^{2+} influx through the NMDA receptor can trigger the activation of the synaptic changes that underlie the processes of learning and memory (Petit, 1988; Bliss and Collingridge, 1993). However, in addition to glutamate, ACh is essential for learning and memory (Winkler et al., 1995), and neuronal nAChRs, particularly the α-BGT-sensitive hippocampal nAChRs, have a high relative permeability to Ca^{2+}. The $P_{Ca/Na}$ for the α-BGT-sensitive neuronal nAChR is about 6, whereas that for the NMDA receptors is approximately 10 (Castro and Albuquerque, 1995). Hence, the activity of neuronal nAChRs could also contribute to control the intracellular levels of Ca^{2+} underlying the changes that account for synaptic plasticity. A number of findings are suggestive of α-BGT-sensitive neuronal nAChRs being located in the dendritic processes of hippocampal neurons: (i) α-BGT-sensitive, fast-desensitizing nicotinic currents can be activated by local application of nicotinic agonists to the dendritic processes of pyramidal neurons (Fig. 1); (ii) rhodamine-labeled α-BGT can bind not only to the soma of cultured hippocampal neurons but also to the dendrites of these neurons (Alkondon and Albuquerque, 1993); and (iii) [^{125}I]α-BGT can label postsynaptic dendritic regions of hippocampal neurons (Hunt and Schmidt, 1978). Taking into account that both cholinergic and glutamatergic synapses can be made on a single dendritic spine (Frotscher, 1992), and that dendritic spines may constitute the anatomical substrate for learning and memory, the hypothesis is raised that a fine and well defined integration of signals coming from different chemical synapses at the level of the spines may be a crucial step in the modulation of cell function and brain activity. To understand such an integration of different neurotransmitter systems in the CNS, it is crucial to characterize the properties of the various receptors, including the mechanisms by which their function can be modulated.

Modulation of the activity of α-BGT-sensitive neuronal nAChRs by ions and by allosteric ligands

Divalent cations modulate the activation of α-BGT-sensitive neuronal nAChRs

As previously reported, extracellular Ca^{2+} can control the activation of α-BGT-insensitive neuronal nAChRs (Mulle et al., 1992). However, it was not until very recently that we have demonstrated that extracellular Ca^{2+} plays a critical role in the regulation of the activation of the α-BGT-sensitive neuronal nAChRs in hippocampal neurons. Extracellular Ca^{2+} controls the rectification, and the rates of desensitization and rundown of type IA currents, as well as the affinity of the α-BGT-sensitive nAChR for ACh and the cooperativity between the ACh binding sites at this receptor (Castro and Albuquerque, 1995; Bonfante-Cabarcas et al., 1995, 1996).

We have shown that the activation of α-BGT-sensitive nAChRs can also be controlled by intracellular Mg^{2+} (Alkondon and Albuquerque, 1993; Alkondon et al., 1994). When a Mg^{2+}-containing internal solution is used, type IA currents recorded from hippocampal neurons or from neurons of the olfactory bulb show a very strong inward rectification (Alkondon and Albuquerque, 1993; Alkondon et al., 1994). In fact, the intracellular Mg^{2+}-induced inward rectification of currents elicited by activation of α7-based nAChRs was recently confirmed in studies carried out in oocytes ectopically expressing these receptors (Forster and Bertrand, 1995). We have now demonstrated that this intracellular Mg^{2+}-induced inward rectification of type IA currents is modulated by extracellular Ca^{2+}. When nominally Mg^{2+}-free, malate-based internal solution was used and the extracellular solution contained 2 mM Ca^{2+}, the rectification of type IA currents was confined to a short range of membrane potentials (0–30 mV). Upon raising the intracellular concentration of Mg^{2+} to 10 mM, the inward rectification persisted up to 50 mV, and if concomitantly the $[Ca^{2+}]_o$ was lowered to 0.3 mM or less, the rectification persisted up to 70 mV (Fig. 3). Therefore, in the presence of low levels of extracellular Ca^{2+}, the activity of the α-BGT-sensitive nAChR at the depolarized membrane potentials would be negligible. This intracellular Mg^{2+}-induced inward rectification of type IA currents, which is intensified by decreasing $[Ca^{2+}]_o$, may be very relevant to a possible functional integration of cholinergic and glutamatergic inputs onto a dendritic spine.

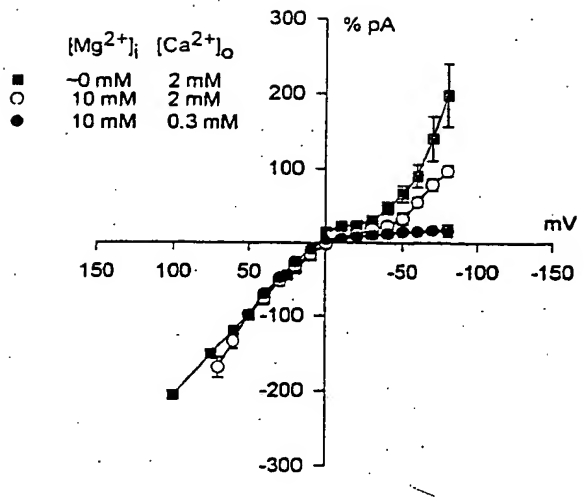

Fig. 3. Effects of extracellular Ca^{2+} on the Mg^{2+}-induced inward rectification of type IA currents. After correction for the rundown, the peak amplitude of the ACh (1-s pulse, 1 mM)-evoked currents to hippocampal neurons held at various holding potentials was normalized to that of the currents evoked at −50 mV. Three different experimental conditions were tested: ■, 2 mM Ca^{2+}-containing external solution and nominally Mg^{2+}-free, malate-based internal solution; ○, 2 mM Ca^{2+}-containing external solution and malate-based internal solution containing 10 mM Mg^{2+}; ●, 0.3 mM Ca^{2+}-containing extracellular solution and malate-based internal solution containing 10 mM Mg^{2+}. The methodology used to record the whole-cell currents was the same as that described in Fig. 1, except that an inverted light microscope was used to visualize the neurons and mechanical micromanipulators were used to hold the patch pipettes and the U-tube. For further details see Bonfante-Cabarcas et al. (1995b).

Similarly to the NMDA receptor, the α-BGT-sensitive nAChR is highly permeable to Ca^{2+}. Thus, if both the NMDA receptors and the α-BGT-sensitive nAChRs were located close to one another on some of the spines known to receive both cholinergic and glutamatergic inputs

(Frotscher, 1992), one would expect that upon simultaneous activation of the cholinergic and glutamatergic terminals, these spines would be flooded with Ca^{2+}. Such an overload is unlikely to happen because according to some studies extracellular Mg^{2+} inhibits the activation of the NMDA receptors at negative membrane potentials (Mayer et al., 1984) and potentiates the activation of these receptors at positive potentials (Wang and McDonald, 1995), and as described here intracellular Mg^{2+} inhibits the activation of the α-BGT-sensitive nAChRs at positive membrane potentials (Alkondon et al., 1994). Whereas the physiological levels of intracellular Mg^{2+} may be enough to cause the inward rectification of α-BGT-sensitive, type IA currents, the increase in the levels of intracellular Mg^{2+} that accompanies the activation of the NMDA receptors (Brocard et al., 1993) would guarantee that the α-BGT-sensitive neuronal nAChRs would remain inactive under conditions at which the NMDA receptors are fully operational.

The rundown of type IA currents, i.e. the progressive decrease of the peak current amplitude with the recording time, has been shown to be dependent upon the metabolic state of the neurons. By adding ATP-regenerating compounds to the intracellular solution, this rundown can be prevented to a great extent (Alkondon et al., 1994). Recently, we have provided evidence that if F^- is replaced with malate in the internal solution, and concomitantly the extracellular concentration of Ca^{2+} is decreased to 0.3 μM or less, the rate of rundown is substantially prolonged, in this way suggesting that the rate of rundown is controlled by the metabolic state of the neurons and to the extracellular levels of Ca^{2+}(Bonfante-Cabarcas et al., 1995a,b).

Changes in the $[Ca^{2+}]_o$ also altered the affinity of the α-BGT-sensitive neuronal nAChR for ACh, and the cooperative interactions between the agonist binding sites on this receptor. At 10 μM $[Ca^{2+}]_o$, the values of EC_{50} and Hill coefficient for ACh in eliciting type IA currents were $233 \pm 13\,\mu$M and 2.63 ± 0.46, respectively, whereas at 1 mM $[Ca^{2+}]_o$ these values were $174 \pm 2\,\mu$M and 1.39 ± 0.14, respectively. Increasing the $[Ca^{2+}]_o$ from 2 to 10 mM altered both the EC_{50} and the Hill coefficient for ACh, as well as the rate of decay of type IA currents (Bonfante-Cabarcas et al., 1995, 1996; Castro and Albuquerque, 1995). In the presence of 10 mM $[Ca^{2+}]_o$, the EC_{50} and the Hill coefficients for ACh were 291 ± 4 and 1.00 ± 0.12, respectively, and the decay phase of the currents had a time constant of about 10 ms (in contrast to the decay-time constant of about 20 ms when $[Ca^{2+}]_o$ was 2 mM). The acceleration of the decay phase of type IA currents observed when the $[Ca^{2+}]_o$ was increased from 2 to 10 mM was indicative of extracellular Ca^{2+} playing an important role in the rate of desensitization of the α-BGT-sensitive nAChRs.

In addition to Ca^{2+} and Mg^{2+}, Pb^{2+} can modulate the activation of both NMDA receptors and α-BGT-sensitive neuronal nAChRs (Alkondon et al., 1990; Guilarte and Micele, 1992; Ujihara and Albuquerque, 1992a; Ishihara et al., 1995a,b). These effects of Pb^{2+}, some of which are developmentally regulated, may indeed be very important for the toxic actions of this heavy metal (Alkondon et al., 1990; Bressler and Goldstein, 1991; Guilarte and Miceli, 1992). Applying the whole-cell mode of the patch-clamp technique to hippocampal neurons either cultured or acutely dissociated from the brain of 3–30-day-old rats, we have shown that Pb^{2+} acts as a non-competitive inhibitor of the activation of α-BGT-sensitive nicotinic currents; the IC_{50} for Pb^{2+} was found to be about 3 μM (Ishihara et al., 1995a). The blocking action of Pb^{2+} is selective for the α-BGT-sensitive nAChR, because only at higher concentrations would Pb^{2+} inhibit the activation of the DHβE-sensitive nAChR in hippocampal neurons, and practically no inhibition of the activation of the muscle nAChR could be observed in the presence of Pb^{2+} (Atchison and Narahashi, 1984; Ishihara et al., 1995a). Similarly, Pb^{2+} inhibits the activation of the NMDA-type of glutamate receptor without affecting other ionotropic types of glutamate receptors (Alkondon et al., 1990; Ujihara and Albuquerque, 1992a; Ishihara et al., 1995b).

We have reported that hippocampal neurons can respond to co-application of NMDA and glycine with whole-cell currents that have a rapidly and a slowly desensitizing component (Ujihara and Al-

buquerque, 1992b; Ishihara et al., 1995b). The fast-desensitizing component, which is the major portion of the NMDA-evoked currents recorded from hippocampal neurons cultured for 3–10 days or from neurons acutely dissociated from the hippocampus of 3–10-day-old rats, is very sensitive to the blockade by Pb^{2+}. In contrast, the slowly desensitizing component, which is the major portion of the NMDA-evoked currents recorded from hippocampal neurons cultured for more than 15 days or from neurons acutely dissociated from the hippocampus of 15–30-day-old rats, is much less sensitive to the inhibitory action of Pb^{2+} (Ujihara and Albuquerque, 1992a; Ishihara et al., 1995b). Several reports support the concept that each of these responses is subserved by a specific NMDA receptor subtype (Tsumoto et al., 1987; Hestrin, 1992; Kutsuwada et al., 1992; Monyer et al., 1992, 1994; Carmignoto and Vicini, 1993; Petralia et al., 1994), and that the expression of such distinct NMDA receptor subtypes is developmentally regulated in hippocampal neurons (Monyer et al., 1994).

At 10 μM, Pb^{2+} decreases by about 50% the peak amplitude of the fast-desensitizing NMDA-evoked currents in immature neurons, whereas at the same concentration Pb^{2+} decreases by only 10% the peak amplitude of the slowly desensitizing NMDA responses in mature neurons (Ujihara and Albuquerque, 1992a). The findings that the inhibitory effect of Pb^{2+} on the NMDA receptor can be antagonized competitively by Ca^{2+} (Fig. 4; see also Marchioro et al., 1995) led to the conclusion that Pb^{2+}-induced inhibition of the activation of the NMDA receptors is mediated by its binding to an extracellular Ca^{2+} site located at the NMDA receptor. Also, although Pb^{2+} is not a competitive inhibitor of NMDA or glycine at the NMDA receptor, glycine modulates the actions of Pb^{2+} on the NMDA receptor. In the presence of low concentrations of glycine, Pb^{2+} (10 μM) was unable to decrease the peak amplitude of the NMDA-evoked currents. Instead, in the presence of nanomolar concentrations of glycine, Pb^{2+} increased the total peak current amplitude and the amplitude of the

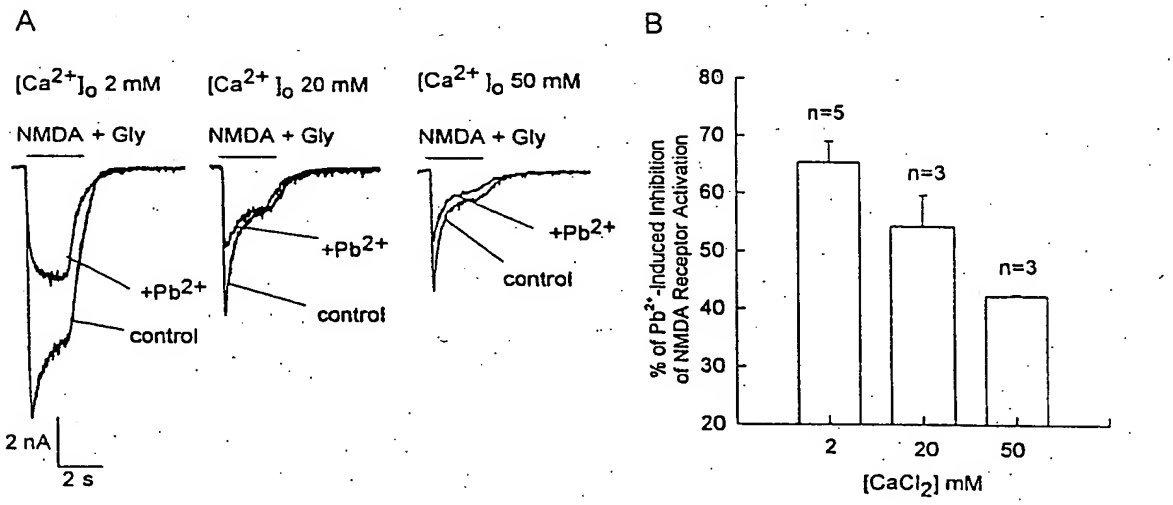

Fig. 4. Pb^{2+}-induced inhibition of NMDA-elicited currents is dependent upon $[Ca^{2+}]_o$. (A) Representative traces of whole-cell currents elicited by fast application of NMDA (50 μM) plus glycine (10 μM) to neurons bathed in external solution containing different $[Ca^{2+}]_o$ in the presence or in the absence of 30 μM Pb^{2+} (Pb^{2+} was added to the bathing solution and to the agonist-containing solution). Increasing the $[Ca^{2+}]_o$ reduced the NMDA response and decreased the potency of Pb^{2+}. Holding potential, –60 mV. Bars above the traces represent the duration of the agonist application. The magnitude of the blocking effect of Pb^{2+} under different experimental conditions is shown in (B). The methodology used to record the whole-cell currents was the same as that described in Fig. 1, except that an inverted light microscope was used to visualize the neurons and mechanical micromanipulators were used to hold the patch pipettes and the U-tube. For further details see Marchioro et al. (1995).

slowly decaying component of the NMDA-elicited current (Marchioro et al., 1995). In the presence of 50 μM kynurenic acid, a specific antagonist of glycine at the NMDA receptor, the potentiating effect of Pb^{2+} was no longer observed. Under this condition, Pb^{2+} inhibited the activation of the NMDA receptors. These novel findings support the notion that the potentiation of the NMDA-evoked responses by Pb^{2+} are due to its binding to the glycine site on the NMDA receptors (Marchioro et al., 1995). Given that extracellular Ca^{2+} also controls the function of the α-BGT-sensitive nAChRs in hippocampal neurons, it remains to be determined whether the inhibitory action of Pb^{2+} on this nAChR subtype is due to the binding of this cation to the same allosteric sites to which Ca^{2+} binds on the nAChR.

Ligand-controlled modulation of nAChR activity

A number of ligands have been shown to modulate allosterically the activation of neuronal nAChRs. For instance, the steroids progesterone and testosterone at low micromolar concentrations can inhibit the activation of the $\alpha4\beta2$ neuronal nAChR ectopically expressed in *Xenopus* oocytes (Valera et al., 1992). Also, (i) the tricyclic antidepressants imipramine and desipramine at submicromolar concentrations inhibit the activation of the neuronal nAChRs expressed in the neuroblastoma cell line SH-SY5Y (Rana et al., 1993), (ii) MK-801, a drug believed to be a specific inhibitor of the activation of NMDA receptors, can inhibit the activation of neuronal nAChRs expressed on retinal ganglion cells (Ramoa et al., 1990). The potency of these ligands as negative modulators of the activation of neuronal nAChRs is comparable to their potency as inhibitors of the activation of other receptor types, particularly the NMDA receptors, indicating that simultaneous modulation of the activation of various CNS receptor types by a single ligand should be considered when such a drug is used clinically or in experimental designs.

Electrophysiological studies carried out in our laboratory have shown that amantadine, a drug used to treat patients with Parkinson's disease and previously reported to inhibit the activation of NMDA receptors (Kornhuber et al., 1991) and of the muscle nAChRs (Albuquerque et al., 1978), can also inhibit the activation of neuronal nAChRs (Matsubayashi et al., 1995). Under equilibrium conditions, the IC_{50} for amantadine in inhibiting the activation of these currents is about 10 μM (Matsubayashi et al., 1995), a value similar to the K_i for amantadine in inhibiting the binding of MK-801 to the NMDA receptors (Kornhuber et al., 1991). The inhibitory effect of amantadine on the α-BGT-sensitive nAChR is voltage dependent; the effect of amantadine is more intense at more negative membrane potentials, suggesting that amantadine acts as an open-channel blocker of the α-BGT-sensitive nAChR. We have also observed that amantadine can act as an open-channel blocker at the nAChRs that account for type II and type III currents in the hippocampal neurons. In fact, amantadine is apparently much more potent in inhibiting the activation of type III currents than in inhibiting the activation of type II and the IA currents, thus indicating that the $\alpha3\beta4$ nAChRs (which presumably subserve type III currents) are much more sensitive than the $\alpha7$-bearing nAChRs (which account for type IA currents) and the $\alpha4\beta2$ nAChRs (which presumably subserve type II currents) to the inhibition by amantadine (Matsubayashi et al., 1995).

Protection of CNS neurons against neurodegeneration may be the key concept to the treatment of Parkinson's disease and of other neurological diseases. It has been suggested that alterations of the glutamatergic system may be implicated in the neurodegeneration that leads to the symptoms observed in Parkinson's disease (Levy and Lipton, 1990; Meldrum and Garthwaite, 1990). However, Parkinson's disease is also characterized by substantial loss of nAChRs, particularly in the pars compacta of the substantia nigra in the midbrain, and such receptor loss appears to be closely related to the primary histopathological changes observed in Parkinson's disease, i.e. loss of dopaminergic cells in the substantia nigra (Whitehouse et al., 1988; James and Nordberg, 1995; Perry et al., 1995). Considering that the activation of the NMDA receptors and of the neuronal nAChRs can be inhibited by low micromolar concentrations of

amantadine, and that the concentration of amantadine achieved in the brain of patients with Parkinson's disease 90 min after oral administration of the drug is approximately 100 μM (Dannysz et al., 1994), it is likely that this drug acts simultaneously on both the glutamatergic neurotransmission mediated by NMDA receptors and on cholinergic neurotransmission mediated by neuronal nAChRs. In light of these findings, it is tempting to speculate that the inhibition of the NMDA receptors by amantadine may contribute to its beneficial effects on patients with Parkinson's disease, whereas the inhibition of the neuronal nAChRs by this drug would explain, at least in part, some of the side effects that develop as a consequence of amantadine therapy.

One of the most provocative new findings in the context of the modulatory control of the nAChR activation is that compounds such as the anticholinesterases physostigmine and galanthamine and the opioid codeine could modulate the activation of neuronal and non-neuronal nAChRs by binding to a newly identified site on these receptors (Kuhlmann et al., 1991; Okonjo et al., 1991; Pereira et al., 1993a,b, 1994; Schrattenholz et al., 1993a,b, 1996; Maelicke et al., 1995a,b). It was initially reported that these compounds could evoke nicotinic single-channel currents in a variety of preparations (Shaw et al., 1985; Pereira et al., 1993a; Schrattenholz et al., 1993a,b; Storch et al., 1995). The agonist effect of these compounds, although insensitive to the inhibition by competitive nicotinic antagonists, is sensitive to the inhibition by the nAChR-specific monoclonal antibody FK1. Using radiolabeled physostigmine, we have shown that the site to which physostigmine and physostigmine-like compounds bind is close to, but distinct from, the ACh-binding sites on the nAChRs. This site is primarily located at the region including and surrounding the residue Lys-125 of the nAChR α subunits, the region of the nAChR α subunits that represents the epitope for FK1 (Schröder et al., 1994). Even though physostigmine, galanthamine, and codeine were seen to activate nicotinic single-channel currents, these compounds were unable to evoke whole-cell currents (Pereira et al., 1993a,b; Storch et al., 1995). It was then suggested that these compounds were unable to evoke a whole-cell response because (i) the concentrations at which they could activate the nAChR channels overlapped the concentrations at which they could act as non-competitive blockers on the nAChRs, and/or (ii) the probability of the nAChR channels being in the open state was very low when physostigmine and physostigmine-like compounds were used as the agonists. More recently, we demonstrated that galanthamine can potentiate the nicotinic responses evoked by application of nicotinic agonists to PC12 cells (Storch et al., 1995) and to cultured hippocampal neurons (Fig. 5; see also Albuquerque et al., 1995c; Schrattenholz et al., 1996). The finding of an endogenous ligand that can modulate the nAChR activity by binding to this newly discovered "physostigmine-binding site" and of a specific competitive antagonist of this ligand would improve our understanding of the nAChR functions in vivo. Our recent findings that 5-HT and codeine can mimic the effects of physostigmine and of physostigmine-like compounds on nAChR activity (Schrattenholz et al., 1996; Storch et al., 1995) suggested that 5-HT and endogenous opiates could modulate the nAChR activity in vivo by binding to this newly identified site on the nAChRs. The positive modulation of the nAChR activity by physostigmine-like compounds resembles the positive modulation of the GABA receptors by benzodiazepines (Majewska, 1992). It is most likely that multiple modes of modulatory control of receptor activity are related to the higher functions of the mammalian brain, including integrative processes, cognition, and associative memory.

Concluding remarks

Our present knowledge of brain function is still very much limited not only by the complexity of the neuronal wiring in the CNS but also by the enormous diversity of local and integrated mechanisms of control of the function of the great variety of neurotransmitter receptors. Even though it was long known by the scientists working with neuronal circuitry that nerve terminals containing the same type of neurotransmitter can make mor-

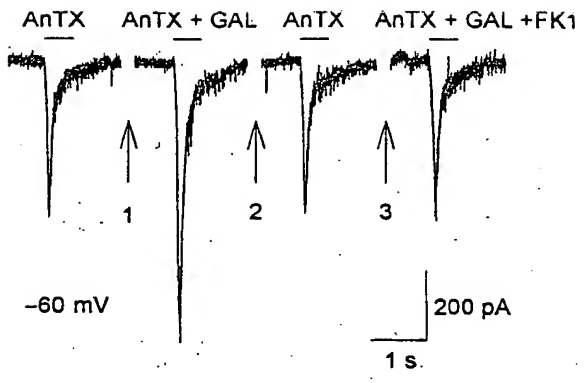

Fig. 5. Potentiation by GAL of AnTX-evoked α-BGT-sensitive nicotinic currents in cultured hippocampal neurons. Effects of GAL (1 μM) on α-BGT-sensitive currents evoked by a 500-ms pulse application of AnTX (10 μM) via a 200-μm aperture on the apex of a U-tube to a cultured hippocampal neuron. The first response of the cells to AnTX (10 μM) was recorded about 15 min after the patch was obtained, at which time the rate of rundown of the peak current amplitude was negligible. Immediately after the control response was obtained (left), the cell was superfused with GAL (1 μM)-containing external solution, the latter being applied via a straight tube that was co-assembled with the U-tube (arrow 1). Under these conditions, the potentiating effect of GAL was observed within 1 min after the start of the perfusion (second from left). When the neuron was then superfused for 2 min with GAL-free external solution (arrow 2), the amplitude of the AnTX-evoked current returned to the prior level (third from left). Finally, the neuron was superfused for 14 min with an FK1 (dilution 1:100)-containing external solution, followed by 1-min perfusion with external solution containing GAL (1 μM) and FK1 (dilution 1:100) (arrow 3), after which time the current evoked by an admixture of AnTX (10 μM), FK1 (dilution 1:100) and GAL (10 μM) was recorded from the cell (right). Notice that pre-incubation of the cells with FK1 prevented the potentiating effect of GAL. The methodology used to record the whole-cell currents was the same as that described in Fig. 1, except that an inverted light microscope was used to visualize the neurons and mechanical micromanipulators were used to hold the patch pipettes and the U-tube. For further details see Schrattenholz et al. (1996).

phologically different synapses onto the same postsynaptic neuron, and that a given dendritic spine could receive inputs of various presynaptic terminals each containing different neurotransmitters, it was not until recently that the diversity of many neurotransmitter receptors (e.g. glutamate receptors, GABA receptors, neuronal nAChRs, and 5-HT receptors) could be proven functionally, biochemically, and using molecular biology techniques.

It is apparent that the function of neuronal chemical networks in the CNS would be tightly controlled if the activity of one postsynaptic receptor was modulated by the activity of another receptor or if a given substance could act simultaneously as the primary agonist of a postsynaptic receptor and as an allosteric modulator of the activation of another postsynaptic receptor. As we discussed throughout this paper, several lines of evidence indicate that both modulatory mechanisms take place in the CNS. Therefore, considering the diversity of the neurotransmitter receptors and their binding sites and the diversity of substances that can simultaneously act as a primary agonist of one receptor and an allosteric modulator of a different receptor, an enormous variety of combinatorial possibilities can be achieved in the brain giving rise to very complex neuronal networks.

The characterization of the diversity of many receptors in the CNS, in addition to the very recent findings that (i) functional neuronal nAChRs and NMDA receptors are expressed on the soma and on the dendrites of hippocampal neurons (Alkondon et al., 1995; Spruston et al., 1995), (ii) Ca^{2+}, Mg^{2+}, Pb^{2+}, and drugs such as amantadine, tricyclic antidepressants, and the anticonvulsant MK-801 can allosterically control the activation of both neuronal nAChRs and NMDA receptors (Mulle et al., 1992; Alkondon et al., 1994; Bonfante-Cabarcas et al., 1996; Marchioro et al., 1995; Matsubayashi et al., 1995), (iii) a single substance can act simultaneously as the primary agonist of one receptor type and as an allosteric modulator of another receptor (Johnson and Ascher, 1992; Garcia-Colunga and Miledi, 1995; Schrattenholz et al., 1996), and (iv) the activity of one receptor type can control the activity of a different receptor type (Medina et al., 1994) may provide the basis for a better understanding of cell function, and, consequently, of synaptogenesis, neuronal development,

and neuronal plasticity that take place under physiological and pathological conditions.

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